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Canadian Journal of Botany

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VOLUME 34 NOVEMBER 1956 NUMBER 6

SAFFLOWER LEAF TISSUE CULTURES[1]

BY FRANZISKA L. M. TUREL AND MARY M. HOWES

Abstract

A tissue culture was obtained from the cells around the vein of a piece of normal safflower leaf. The tissue has now been transferred monthly for two and one-half years. Growth was measured on White's, Knop's, and Heller's media with and without 0.1 mgm. indole-3-acetic acid per liter and/or 10% coconut milk. Indole-3-acetic acid had no effect but coconut milk greatly enhanced growth. Heller's medium plus coconut milk was the best for growth of the leaf tissue. The addition of coconut milk to White's medium caused a decrease in percentage of dry weight of the leaf tissue culture, whereas its addition to Heller's medium had no such effect.

Reports from the literature indicate that true plant tissue cultures have been obtained only from healthy root or stem tissues which contain many meristematic cells, or from tumorous gall tissue (2, 5, 8). In this study true tissue cultures were obtained from the secondary leaves of safflower plants, *Carthamus tinctorius*, variety "Israel".

The work was originally started with rust-infected leaves of wheat, sunflower, flax, and safflower. Since Morel (6) had been unsuccessful in obtaining infections of various plant tissue cultures with "sterile" spores of the corresponding rusts, we attempted to obtain active rust growth on tissue cultures by culturing leaves which had been infected with rust in the greenhouse. The leaves were removed from the infected plant when they showed whitish spots of incipient pustules on the surface. In most trials uredospores broke through the epidermis after the leaves were placed on media in test tubes. Some of the leaves produced new and actively growing callus tissue as described below, but the rust did not spread into the new young growth, and died after several weeks together with the part of leaf tissue which nourished it. Thus cultures were obtained free from the fungus. This investigation deals with some nutritional effects on these leaf tissue cultures.

[1]*Manuscript received June 15, 1956.*
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as N.R.C. No. 4061.

[The September number of this journal (Can. J. Botany, 34 : 745-823. 1956) was issued October 22. 1956.]

Materials and Methods

Media

The media used were White's (7, p. 103), and Heller's (3, pp. 140 and 154). The composition of the latter is given below:

Per liter of solution:

$NaNO_3$	600 mgm.	H_3BO_3	1 mgm.
KCl	750 "	$MnSO_4.4H_2O$	0.1 "
$CaCl_2.2H_2O$	75 "	$CuSO_4.5H_2O$	3×10^{-2} mgm.
$MgSO_4.7H_2O$	250 "	$AlCl_3$	3×10^{-2} "
$NaH_2PO_4.H_2O$	125 "	$NiCl_2.6H_2O$	3×10^{-2} "
$FeCl_3.6H_2O$	1 "	KI	1×10^{-2} "
$ZnSO_4.7H_2O$	1 "		

A single run was made on Knop's medium (1, p. 51).

Where indicated, 0.1 mgm. per liter of indole-3-acetic acid and/or 10% coconut milk were added. The solid media contained 0.8% agar.

Methods

The leaves of plants about 6 in. tall were cut into three or four pieces each about one centimeter square. The pieces were placed in 3% "Javex" solution (containing a few drops "Tween-80") for 10 min., then rinsed in sterile water for 10 min., and placed in liquid medium in test tubes. Each tube contained one piece of leaf and 5 ml. of White's medium with indole-3-acetic acid and fresh coconut milk added. The tubes were set upright under lights* and incubated at 20° to 25°C. By six weeks, greenish growth appeared at the ends of the veins in the leaf portions in only two cultures of the 35 set up. These two cultures were cut into small pieces about 200 to 500 mgm. each, and transferred to fresh liquid medium and to agar medium (White's). The cultures on agar continued to grow well, while those on liquid became brown and died. All subcultures were derived from one of these agar cultures and have been transferred once a month on various agar media.

The new tissue was composed of loose, disorganized, moist callus material, usually very soft and crumbly (Fig. 1), with phloem cells present. No buds or roots were formed. Its color was yellowish-green, although during the first two years some of the cultures on Heller's medium were bright green and firmer. This bright green was not retained in repeated subculture during two and a half years while growth of the pale cultures is still vigorous. Cultures on White's medium never looked as healthy as on Heller's medium and always had a more brownish tinge.

Once a month the cultures were broken up into small pieces (200 to 500 mgm.), transferred to fresh media, and pressed slightly into the agar. At least three transfers to the same medium were made for each culture before it was measured for comparative experiments.

**Westinghouse Standard Cool White Fluorescent Light, about 500 ft-c.*

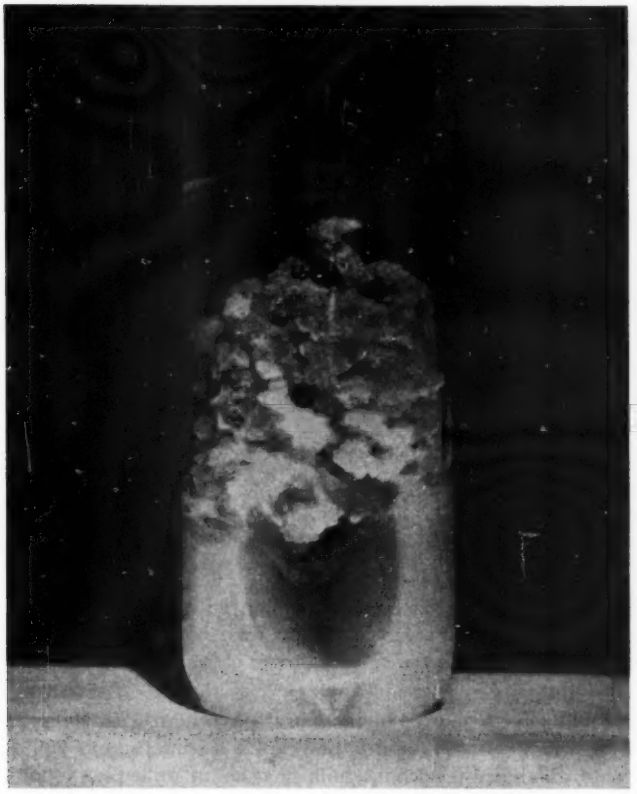

FIG. 1. Safflower leaf tissue culture on Heller's medium containing 10% coconut milk.

Heller's method (3, pp. 42–43) for measuring the amount of growth of the cultures on the various media was used. Small paper envelopes were sterilized, the culture transferred to them, and each weighed singly on a balance. The culture was then removed to a sterile Petri dish and the envelopes weighed alone. Very seldom were any contaminants introduced. The growth increase in terms of percentage of the initial weight was obtained by dividing the amount of growth in one month by the initial weight times 100.

To determine the dry weights of cultures which had been transferred at least three times on the same agar medium, the callus pieces were predried singly on Petri plates at 60°C. until they were brittle, and they were then vacuum-dried. The amount of dry weight was expressed in percentage of fresh weight. The values in Table I refer to weighings of cultures which had been transferred monthly for about a year and a half.

TABLE I

GROWTH OF SAFFLOWER LEAF TISSUE CULTURES ON DIFFERENT AGAR MEDIA

Medium	Increase in per cent of initial fresh weight. Monthly transfers†				Per cent dry weight
	1	2	3	Average	
White's	129	49	56	78	6.7 ± 1.1**
White's + IAA‡	83	58	58	67	6.9 ± 1.5**
White's + IAA + CM‡	341	242	211	265	5.2 ± 0.5*
Heller's	265	259	188	237	5.6 ± 2.2
Heller's + IAA	214	276	165	218	6.2 ± 2.3
Heller's + CM	404	413	320	379	6.0 ± 1.5
Heller's + IAA + CM	424	376	228	343	6.5 ± 1.4

†*Each value represents an average of the weights of 9 to 13 cultures.*
‡IAA *is 0.1 mgm. per liter indole-3-acetic acid and CM is 10% coconut milk.*
NOTE: *The difference between * and ** is significant at the 1% level.*

Results and Discussion

As shown in Table I, Heller's medium promoted much better growth of the safflower leaf tissue cultures than did White's medium. Indole-3-acetic acid had no noticeable influence, either as an addition to the basic media alone or added together with coconut milk.

A single run was made on Knop's medium with agar and indole-3-acetic acid. There the average increase in weight was 166% over the initial weight, which is between the average weight increases obtained with Heller's and with White's medium.

Coconut milk gave a marked increase in fresh weight; with Heller's medium the increase was about 60% and with White's medium about 250%. Since there was no difference if coconut milk or coconut milk plus indole-3-acetic acid were added to Heller's solution, it was assumed that the same would be true with White's medium; therefore no trial was made with White's plus coconut milk alone.

In all the series shown in Table I it seems that, from the first to the third transfer, growth of the cultures decreased more or less regularly. This observation was also made in different series several months apart but proved to be incidental, since a successive and continued decrease in growing power of the cultures has not been observed. To facilitate comparison between the different experimental series, averages of the three transfers are also given.

Measurements of dry weight of the cultures were carried out because Hildebrandt and Riker (4) found an influence of some growth factors on dry weight of their sunflower and tobacco tissue. They state that "the lowest relative dry weight appeared at those concentrations where the greatest wet weight occurred". In our experiments this seemed to be true for the great stimulation of growth when coconut milk was added to White's medium. Here we obtained a highly significant decrease in dry weight by adding coconut

milk. However, when the latter was added to Heller's medium the increase in growth caused by coconut milk was not accompanied by a decrease in percentage of dry matter, which again seems to indicate a better balanced and healthier growth of the tissue on Heller's solution, besides the healthier appearance of the cultures as mentioned above. Over all, Heller's medium was highly satisfactory and seems much better balanced for use with our leaf tissue cultures than White's medium. One disadvantage with Heller's medium was that the standard deviations of the dry weights were quite high but this difficulty did not appear when coconut milk was added.

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A CASE OF SOMATIC REDUCTION IN A PREMEIOTIC CELL IN WHEAT[1]

By D. R. Knott[2]

Abstract

An unusual case of somatic reduction was found at first meiotic metaphase in a single anther of a wheat plant. Of 10 reduced cells, four had 22 chromosomes, five had 20, and one was not clearly countable. Chromosome pairing indicated that the reduction had involved a random segregation of homologues.

Hollingshead (1) and Love (2, 3) have reported the occurrence at first meiotic metaphase in wheat of cells having a reduced number of chromosomes. The cells had varying chromosome numbers, and were frequently found in plants derived from an interspecific cross.

A new and somewhat unusual case of cells having approximately the haploid number of chromosomes has been observed. The plant involved came from the first backcross to Thatcher of a stable wheat–*Agropyron* derivative (Chinese Spring × *Agropyron elongatum* backcrossed to Chinese Spring). In an acetocarmine smear of a single anther from this plant, five cells at metaphase I were found to have 20 chromosomes and four had 22 chromosomes. A 10th cell was broken but probably had 22 chromosomes. All of the remaining cells had 42 chromosomes and usually formed 21 bivalents, although several cells had 20 bivalents and two univalents. The pairing behavior of the 10 reduced cells is shown in Table I and four of the cells are pictured in Figs. 1–4.

Since all of the exceptional cells had either 20 or 22 chromosomes, it appears probable that they traced back to the products of a single somatic reduction. That this assumption is true is shown by the fact that not only did the two groups of cells have complementary chromosome numbers, but also, with one exception, the cells within each group contained identical numbers of closed bivalents. Thus all cells of one chromosome number contained the same number of homologous pairs. In the exceptional 20-chromosome cell it is likely that one homologous pair formed an open rather than a closed bivalent. The other open bivalents probably resulted from the pairing of homeologous chromosomes such as has been shown to occur in a wheat haploid, and their number would therefore be expected to vary from cell to cell.

Assuming that only one reduction occurred, then it is evident that the two original cells were each able to divide at least two or three times despite their unbalanced chromosome complements.

[1]*Manuscript received May 7, 1956.*
Contribution from the Department of Field Husbandry, University of Saskatchewan, Saskatoon, Sask.
[2]*Associate Professor of Field Husbandry, University of Saskatchewan.*

TABLE I

PAIRING BEHAVIOR OF CHROMOSOMES IN CELLS HAVING A REDUCED CHROMOSOME NUMBER

No. of chromosomes	Closed bivalents	Open bivalents	Univalents
20	4	0	12
20	3	1	12
20	4	1	10
20	4	1	10
20	4	3	6
22	5	0	12
22	5	1	10
22	5	2	8
22	5	2	8
?*	5	0	8

**This cell was badly smashed in smearing but on the basis of the number of closed bivalents present probably belonged in the 22 chromosome group.*

Since Huskins emphasized the possible importance of somatic reduction in plants, the question has been raised whether the segregation of chromosomes in somatic reduction is random or involves a separation of homologues. Chromosome pairing in the 10 cells observed in this case shows that the reduction that took place was random and therefore did not involve somatic pairing and separation of homologues. The regular occurrence of five closed bivalents in the 22 chromosome cells and of four closed bivalents in the 20 chromosome cells shows that nine pairs of homologues, or approximately half, segregated into the same cell. This is the result that would be expected if the chromosomes had separated at random into two groups.

References

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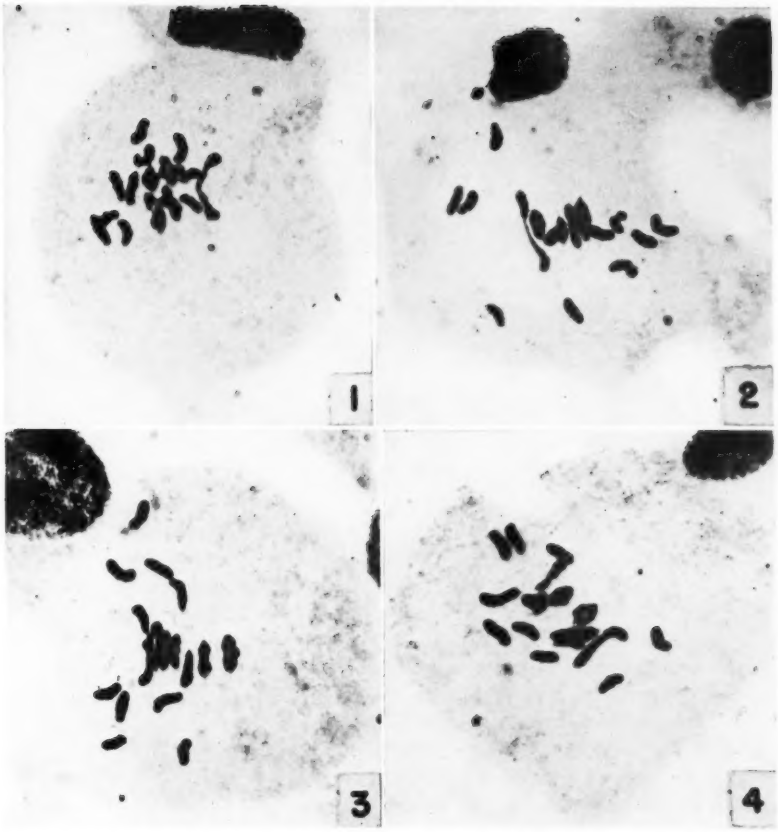
2. LOVE, R. M. Occurrence of haploid pollen mother cells in a *vulgare* wheat. Nature, 138 : 589-590. 1936.
3. LOVE, R. M. Somatic variation of chromosome numbers in hybrid wheats. Genetics, 23 : 517-522. 1938.

PLATE I



FIGS. 1 and 2. Cells showing four closed bivalents, one open bivalent, and 10 univalents.
FIGS. 3 and 4. Cells showing five closed bivalents, two open bivalents, and eight univalents.

THE OCCURRENCE OF FUSARIUM SPECIES IN CANADA

V. TAXONOMY AND GEOGRAPHIC DISTRIBUTION OF FUSARIUM SPECIES IN SOIL[1]

By W. L. Gordon[2]

Abstract

A taxonomic study was made of 1348 isolates of *Fusarium* found in 206 soil samples collected in 1946. Approximately 70% of the samples, or 143, were obtained from 127 localities in Manitoba; the remaining 30%, or 63, came from seven other provinces.

Of 25,973 colonies of fungi that developed on the 2060 soil-dilution plates involved in this study, *Fusarium* accounted for approximately 5%. A total of 14 species and varieties of *Fusarium*, classified in nine sections of the genus, were isolated; they are *F. merismoides* Cda., *F. poae* (Pk.) Wr., *F. sporotrichioides* Sherb., *F. avenaceum* (Fr.) Sacc., *F. semitectum* Berk. & Rav., *F. acuminatum* Ell. & Ev., *F. equiseti* (Cda.) Sacc., *F. culmorum* (W. G. Sm.) Sacc., *F. sambucinum* Fuckel, *F. sambucinum* var. *coeruleum* Wr., *F. moniliforme* Sheld. emend. S. & H., *F. oxysporum* Schlecht. emend. S. & H., *F. oxysporum* var. *redolens* (Wr.) Gordon, *F. solani* (Mart.) App. & Wr. emend. S. & H. *F. oxysporum*, *F. oxysporum* var. *redolens*, *F. equiseti*, and *F. solani* accounted for approximately 90% of the total *Fusarium* isolates. *F. oxysporum* was by far the most prevalent species isolated. With the exception of *F. merismoides*, all of the species isolated were found previously to be seed-borne by cereals and to be associated with common root rot of those crops.

Introduction

The results obtained from a previous study (5) of the *Fusarium* flora of the soil of permanent cereal plots at Winnipeg, Man., which were examined annually for several years, showed that this genus comprised on the average about one-sixth of the total fungus flora and consisted of an appreciable number of species, varieties, and forms. As might be expected from the results obtained by previous investigators of the *Fusarium* flora of the soil (7, 9), certain species proved to be much more prevalent than others. For instance, of the total of 16 species, varieties, and forms that were isolated, *F. oxysporum* Schlecht. emend. S. & H. and *F. equiseti* (Cda.) Sacc. together accounted for approximately 75% of the total isolates, the former species alone accounting for approximately 50%. As it was uncertain whether this picture of the *Fusarium* flora, with respect to its proportion of the total fungus flora and to the identity of the species constituting it, was typical of soil generally in Manitoba, the need of a further investigation to elucidate these points was indicated.

The present study was undertaken in 1946 at the Plant Pathology Laboratory, Winnipeg, Man., to ascertain the identity of *Fusarium* isolates that could be obtained from soils of various types and vegetative cover. Most of the soil samples originated in Manitoba, but it was possible to examine as well a relatively small number of samples from each of seven other provinces.

[1]*Manuscript received June 27, 1956.*
Contribution No. 1554 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.
[2]*Senior Plant Pathologist, Plant Pathology Laboratory, Winnipeg, Manitoba.*

Materials and Methods

A total of 206 composite samples of soil were collected in 1946 and were examined for the presence of *Fusarium* by the dilution-plate method. The first of these samples was taken in early June, and others were obtained from time to time until the latter part of October. Approximately 70%, or 143 samples, originated in 127 localities in Manitoba. These Manitoba samples were taken from each of the several soil zones in the province. The remaining 30%, or 63 samples, came from seven other provinces, 17 from Ontario, 13 from Saskatchewan, 12 from Quebec, nine from British Columbia, and four each from Alberta, Nova Scotia, and Prince Edward Island.

Most of the 206 samples collected were taken from various types of soil sustaining a wide variety of cultivated crops or native vegetation. Of 75 samples taken from cereal soils, 38 were taken from fields of wheat, 17 from oats, 13 from barley, four from corn, and three from rye. An additional 47 samples were taken from soils sustaining other field crops, of which 18 were from fields of pasture-grass; 10 from flax; six from alfalfa; four from peas; three from clover; and two from sunflowers, potatoes, and tobacco, respectively. Another group of 31 samples consisted of 19 taken in vegetable and ornamental gardens and 12 in orchards. Of 36 samples taken from soils sustaining native vegetation, 14 were obtained from native grasslands, and 22 from wooded areas. A total of 17 samples were taken from fields in fallow.

The procedure followed in taking the soil samples was as follows: After the loose surface soil or vegetative covering was removed, a sample was taken to a depth of about six inches with a clean spade or trowel and placed in a wax-coated carton or a durable paper bag. Each sample taken in the field for analysis in the laboratory was usually a composite of four smaller samples. Samples taken in Manitoba were somewhat larger than those taken elsewhere but a field sample consisted of at least a pint of soil.

When the samples of soil reached the laboratory they were allowed to become air-dry and were then passed through a 20-mesh sieve to remove any coarse organic matter and gravel present in them. The time required for air-drying the samples varied from two to six days, depending chiefly on the amount of moisture present in the soil at the time of sampling. After each sample had been air-dried and sieved, a portion consisting of 25 gm. was taken from it and suspended in sterile water. A dilution of 1:5000 was employed for each of the samples, as this dilution was found previously to be satisfactory for the isolation of *Fusarium* from soil (5). A total of 10 ml. was taken from each soil dilution, 1 ml. being added to each of 10 Petri plates, after which they were poured with potato sucrose agar, acidified to approximately pH 4, and incubated for seven days at a temperature of from 22° to 24° C. After the period of incubation, the number of fungus colonies that appeared on each of the 2060 Petri plates used in this study were counted and recorded. The colonies of *Fusarium* that developed on the plates were transferred to potato sucrose agar tubes for identification studies.

Prevalence of *Fusarium* in the Soil

Of the total of 25,973 colonies of fungi that developed on the dilution plates, *Fusarium* accounted for 1348, or approximately 5%. This percentage of the total fungus colonies is considerably lower than that accounted for by *Fusarium* in the soil of permanent cereal plots located at Winnipeg, Man., which averaged approximately 16% over a seven-year period (5).

During the present study 19 samples,[3] or approximately 9% of the 206 examined, did not yield *Fusarium* isolates even when re-examined at a dilution of 1:1000. Most of these samples had originated in sandy, wooded soils in Manitoba. It was observed that colonies of *Penicillium* spp. were particularly common on the sets of dilution plates lacking *Fusarium*. This observation suggests the possibility that the apparent absence of *Fusarium* in these soils may be attributed to antibiotic activity on the part of the *Penicillium* species present.

Taxonomy of the *Fusarium* Isolates from Soil

The various wild-type colonies of *Fusarium* obtained from soil were named and classified according to the modified Wollenweber system adopted previously (3, 4, 5). Fourteen species and varieties were identified among the 1348 isolates. These species and varieties represented nine sections of the genus, namely, Eupionnotes, Sporotrichiella, Roseum, Arthrosporiella, Gibbosum, Discolor, Liseola, Elegans, and Martiella.

Four species not isolated from soil during the present study, but reported previously to occur in soil in Canada (1, 5), are not included in the taxonomic part of this paper. These species, and the section of the genus in which they are classified, are *F. dimerum* Penz. (Eupionnotes); *F. concolor* Rg. (Arthrosporiella); *F. sambucinum* Fuckel f. 6 Wr. (Discolor); and *F. coeruleum* (Lib.) Sacc. (Martiella).

The synonymy of the species, varieties, and forms given in this paper is largely restricted to those names that may be encountered in modern mycological and phytopathological literature of *Fusarium*, including Raillo's recent monograph entitled "Grebi roda *Fusarium*" (8), which has been translated as "Fungi of the genus *Fusarium*". Synonyms given previously in Parts II and III of this series of papers (3, 4) have been omitted here. A more comprehensive synonymy can be found in Fusarium-Monographie by Wollenweber (10, 11) and in Die Fusarien by Wollenweber and Reinking (12).

Section Eupionnotes Wr.

Wollenweber, Phytopath. 3 : 206 and 219. 1913.
Wollenweber, Fusarium Monographie, pp. 291–292. 1931.

[3]*Six additional samples of soil examined in 1949 at dilutions of 1:5000, 1:1000, and 1:500 did not yield Fusarium isolates. Five samples originated in the Northwest Territories of which four were collected at Yellowknife and one at Artillery Lake. The remaining sample originated at Bear Creek, Yukon.*

Wollenweber and Reinking, Die Fusarien, pp. 18–20. 1935.
Raillo, Fungi of the genus *Fusarium*, pp. 272 and 276. 1950.

F. merismoides Cda., belonging in this section of the genus, was isolated from soil during this study.

FUSARIUM MERISMOIDES Cda.
Corda, Icones fungorum, II : 4. 1838.
Wollenweber and Reinking, Die Fusarien, pp. 24–25. 1935.
Raillo, Fungi of the genus *Fusarium*, pp. 280–281. 1950.
Gordon, Can. J. Botany, 30 : 215–216. 1952.

Syn. *F. betae* (Desm.) Sacc.
Saccardo, Michelia, 2 : 133. 1880.

The first record of the occurrence of this species in Canada was made in 1924 by Henry (6), who isolated it from soil obtained from Brandon, Man. It was present each year from 1936 to 1942 in the soil of permanent cereal plots at Winnipeg, Man. (5). This species has been isolated also from soil at Ancaster, Ont., in 1950 by R. G. Atkinson (in litt.).

F. merismoides accounted for approximately 1% of the isolates of *Fusarium* during this study. Of the 14 isolates that were obtained from soil, 13 were from Manitoba and one from Saskatchewan. This species is not known to cause plant diseases in Canada.

Section Sporotrichiella Wr.

Wollenweber apud Lewis in Maine Agr. Expt. Sta. Bull. 219 : 256. 1913.
Wollenweber and Reinking, Die Fusarien, pp. 45–47. 1935.
Wollenweber, Fusarium-Monographie II, pp. 125–127. 1944–45.
Raillo, Fungi of the genus *Fusarium*, pp. 192–194. 1950.

Two species, namely, *F. poae* (Pk.) Wr. and *F. sporotrichioides* Sherb., classified in this section of the genus, have been isolated from soil in Canada.

FUSARIUM POAE (Pk.) Wr.
Wollenweber and Reinking, Die Fusarien, p. 47. 1935.
Wollenweber, Fusarium-Monographie II, pp. 127–128. 1944–45.
Raillo, Fungi of the genus *Fusarium*, pp. 194–195. 1950.
Gordon, Can. J. Botany, 30 : 218–219. 1952.

Syn. *F. poae* (Pk.) Wr. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 195. 1950.

F. poae was first isolated from soil in Canada in 1933, from a sample of soil from Manitoba (1). Additional isolates of it were obtained each year from 1936 to 1942, from the soil of permanent cereal plots at Winnipeg, Man. (5).

During the present study eight isolates of *F. poae* were obtained from soil; five were from Manitoba and three from Quebec. Isolates of this species accounted for only a fraction of 1% of the total *Fusarium* isolates.

FUSARIUM SPOROTRICHIOIDES Sherb.

Sherbakoff, N.Y. Cornell Agr. Expt. Sta. Memoir, 6 : 183–186. 1915.
Wollenweber and Reinking, Die Fusarien, pp. 48–49. 1935.
Wollenweber, Fusarium-Monographie II, pp. 129–130. 1944–45.
Raillo, Fungi of the genus *Fusarium*, p. 196. 1950.

This species has previously been found to be uncommon in soil in Manitoba (1, 5). During the present study only two isolates were obtained, both from Manitoba soil.

Section Roseum Wr.

Wollenweber, Phytopath. 3 : 32. 1913.
Wollenweber and Reinking, Die Fusarien, pp. 49–53. 1935.
Wollenweber, Fusarium-Monographie II, pp. 130–131. 1944–45.
Raillo, Fungi of the genus *Fusarium*, pp. 181–185. 1950.

Syn. Subsection Euroseum Raillo
Raillo, Fungi of the genus *Fusarium*, pp. 181 and 185. 1950.

Only one species, namely, *F. avenaceum* (Fr.) Sacc., belonging in this section of the genus, has been isolated from soil in Canada.

FUSARIUM AVENACEUM (Fr.) Sacc.

Saccardo, Syll. Fungorum, 4 : 713. 1886.
Wollenweber and Reinking, Die Fusarien, pp. 53–55. 1935.
Wollenweber, Fusarium-Monographie II, pp. 132–135. 1944–45.
Gordon, Can. J. Botany, 30 : 220–221. 1952.

Syn. *F. avenaceum* (Fr.) Sacc. subsp. *volutum* Raillo
Raillo, Fungi of the genus *Fusarium*, pp. 188–189. 1950.

This species was first isolated from a sample of garden soil in Manitoba in 1933 (1). It was found later to be present in the soil of permanent cereal plots at Winnipeg, Man., during each year of the period 1936–1942. Each year of this period, however, it formed only a fraction of 1% of the total isolates (5).

During the present study this species proved to be exceedingly rare in the samples of soil examined, for only a single isolate of it was obtained from a sample of soil from Quebec.

Section Arthrosporiella Sherb.

Sherbakoff, N.Y. Cornell Agr. Expt. Sta. Memoir, 7 : 161. 1915.
Wollenweber, Fusarium Monographie, pp. 322–324. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 57–58. 1935.
Raillo, Fungi of the genus *Fusarium*, pp. 160–161. 1950.

Syn. Subsection Euarthrosporiella Raillo
Raillo, Fungi of the genus *Fusarium*, p. 160. 1950.

Subsection Pseudodiscolor Raillo
Raillo, Fungi of the genus *Fusarium*, pp. 160–161. 1950.

Subsection Pseudoroseum Raillo
Raillo, Fungi of the genus *Fusarium*, p. 161. 1950.

F. semitectum Berk. & Rav., classified in this section of the genus, was isolated from soil during this study.

FUSARIUM SEMITECTUM Berk. & Rav.
Berkeley, Grevillea, 3 : 98. 1875.
Wollenweber, Fusarium-Monographie, pp. 324–325. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 58–59. 1935.
Raillo, Fungi of the genus *Fusarium*, p. 163. 1950.
Gordon, Can. J. Botany, 30 : 222–223. 1952.

Syn. *F. semitectum* Berk. & Rav. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 163. 1950.

F. semitectum was previously isolated from the soil of cereal plots located at Winnipeg, Man. (5). Only two isolates of this species were obtained from soil during the present study, one from Manitoba, and the other from Ontario.

Section Gibbosum Wr.

Wollenweber, Phytopathology, 3 : 31. 1913.
Wollenweber, Fusarium-Monographie, pp. 328–330. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 61–62. 1935.
Raillo, Fungi of the genus *Fusarium*, pp. 165–170. 1950.
Gordon, Can. J. Botany, 30 : 223–234. 1952.

Syn. Subsection Eugibbosum Raillo
Raillo, Fungi of the genus *Fusarium*, p. 166. 1950.

Subsection Pseudodiscolor Raillo pr. p.
Raillo, Fungi of the genus *Fusarium*, pp. 166 and 180. 1950.

Two species, namely, *F. acuminatum* Ell. & Ev. and *F. equiseti* (Cda.) Sacc., belonging in the section Gibbosum, have been isolated from soil in Canada.

FUSARIUM ACUMINATUM Ell. & Ev.
Ellis and Everhart, Proc. Acad. Sci. Philadelphia, p. 441. 1895.
Wollenweber, J. Agr. Research, 2 : 269–270. 1914.
Gordon, Can. J. Botany, 30 : 224 and 226. 1952.

Syn. *F. scirpi* Lamb. & Fautr. subsp. *acuminatum* (Ell. & Ev.) Raillo
Raillo, Fungi of the genus *Fusarium*, pp. 177–178. 1950.

F. scirpi Lamb. & Fautr. subsp. *acuminatum* (Ell. & Ev.) var. *triseptatum* Raillo
Raillo, Fungi of the genus *Fusarium*, p. 178. 1950.

F. scirpi Lamb. & Fautr. subsp. *acuminatum* (Ell. & Ev.) form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 178. 1950.

F. scirpi Lamb. & Fautr. subsp. *acuminatum* (Ell. & Ev.) form 2 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 179. 1950.

This species has been known to occur in soil in Canada since 1933 when it was isolated from samples of soil from Manitoba (1). It was isolated again each year from 1936 to 1942 from the soil of permanent cereal plots at Winnipeg (5).

During the present study 40 isolates of this species were obtained. These comprised approximately 3% of the total *Fusarium* isolates. One isolate was obtained from soil from British Columbia, one from Alberta, seven from Saskatchewan, 28 from Manitoba, and three from Quebec. *F. acuminatum* is rather commonly found among isolates of fungi from the diseased basal parts of plants in Canada.

FUSARIUM EQUISETI (Cda.) Sacc.

Saccardo, Syll. Fungorum 4 : pp. 707–708. 1886.
Wollenweber, Fusarium-Monographie, p. 330. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 63–64. 1935.
Raillo, Fungi of the genus *Fusarium*, p. 173. 1950.
Gordon, Can. J. Botany, 30 : 223–226. 1952.

Syn. *F. caudatum* Wr.
Wollenweber, J. Agr. Research, 2 : 262. 1914.
Raillo, Fungi of the genus *Fusarium*, p. 179. 1950.

F. caudatum Wr. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 179. 1950.

F. caudatum Wr. var. *filiferum* Raillo
Raillo, Fungi of the genus *Fusarium*, p. 179. 1950.

F. compactum (Wr.) Raillo
Raillo, Fungi of the genus *Fusarium*, p. 180. 1950.

F. equiseti (Cda.) Sacc. subsp. *ossicolum* (Berk. & Curt.) Raillo
Raillo, Fungi of the genus *Fusarium*, p. 175. 1950.

F. equiseti (Cda.) Sacc. subsp. *ossicolum* (Berk. & Curt.) form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 175. 1950.

F. equiseti (Cda.) Sacc. form 1 Raillo (non Wr.)
Raillo, Fungi of the genus *Fusarium*, p. 174. 1950.

F. equiseti (Cda.) Sacc. form 2 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 174. 1950.

F. scirpi Lamb. & Fautr. form 1 Raillo (non Wr.)
Raillo, Fungi of the genus *Fusarium*, p. 176. 1950.

Fusarium equiseti was previously reported to occur in Manitoba soils (1, 2, 5). From 1936 to 1942 it accounted for approximately 25% of the total isolates of *Fusarium* obtained from the soil of permanent cereal plots at Winnipeg. During the present study this species was again found to be relatively common in soil. It formed approximately 13% of the total isolates of *Fusarium*. Of the 170 isolates obtained, 19 were from soil from British Columbia, six from Alberta, 16 from Saskatchewan, 114 from Manitoba, six from Ontario, four from Quebec, and five from Prince Edward Island.

Section Discolor Wr.

Wollenweber, Phytopathology, 3 : 31. 1913.
Wollenweber, Fusarium-Monographie, pp. 346–349. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 69–72. 1935.
Raillo, Fungi of the genus *Fusarium*, p. 197. 1950.

Syn. Section Trichothecioides Raillo
Raillo, Fungi of the genus *Fusarium*, pp. 217–218. 1950.
Subsection Eudiscolor Raillo pr. p.
Raillo, Fungi of the genus *Fusarium*, pp. 197 and 208. 1950.
Subsection Saubinetii Wr.
Raillo, Fungi of the genus *Fusarium*, pp. 198 and 213. 1950.
Subsection Pseudodiscolor, Section Gibbosum, pr. p.
Raillo, Fungi of the genus *Fusarium*, p. 180. 1950.

Five species, varieties, and forms, namely, *F. culmorum* (W. G. Sm.) Sacc., *F. reticulatum* Mont., *F. sambucinum* Fuckel, *F. sambucinum* var. *coeruleum* Wr., and *F. sambucinum* f. 6 Wr. classified in this section have been reported previously to occur in soil in Manitoba (1, 5). During the present study, only *F. culmorum*, *F. sambucinum*, and *F. sambucinum* var. *coeruleum* were obtained from the various soil samples examined.

FUSARIUM CULMORUM (W. G. Sm.) Sacc.
Saccardo, Syll. Fungorum 11 : 651–652. 1895.
Wollenweber and Reinking, Die Fusarien, pp. 79–81. 1935.
Raillo, Fungi of the genus *Fusarium*, p. 212. 1950.
Gordon, Can. J. Botany, 30 : 227–228. 1952.

Syn. *F. discoloriformis* Raillo
Raillo, Fungi of the genus *Fusarium*, p. 180. 1950.
F. sambucinum Fuckel var. *cereale* (Cke.) Raillo
Raillo, Fungi of the genus *Fusarium*, p. 211. 1950.

Fusarium culmorum was rarely isolated during this study. Only four isolates were obtained. As 75 of the 206 samples of soil originated in cereal fields, this species does not appear to be commonly widespread in cereal soils. Of the four isolates obtained, one was from a sample of soil from British Columbia, two were from Manitoba, and one from Nova Scotia.

FUSARIUM RETICULATUM Mont.
Wollenweber, Fusarium-Monographie, pp. 350–351. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 73–74. 1935.

Syn. *F. heterosporum* Nees pr. p.
Raillo, Fungi of the genus *Fusarium*, p. 216. 1950.
F. heterosporum Nees form 2 Raillo pr. p.
Raillo, Fungi of the genus *Fusarium*, p. 217. 1950.
F. roseum Lk. emend. Snyder & Hansen pr. p.
Snyder and Hansen, Am. J. Botany, 32 : 663. 1945.

This species was isolated from soil in Manitoba in 1933 (1). It was not isolated from the soil of permanent cereal plots at Winnipeg, Man., from 1936 to 1942 (5) and it was not found during the present study.

FUSARIUM SAMBUCINUM Fuckel

Wollenweber, Fusarium-Monographie, pp. 352–353. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 75–76. 1935.
Raillo, Fungi of the genus *Fusarium*, pp. 208–209. 1950.

Syn. *F. sambucinum* Fuckel form 1 Raillo (non Wr.)
Raillo, Fungi of the genus *Fusarium*, p. 210. 1950.

F. sambucinum Fuckel form 2 Raillo (non Wr.)
Raillo, Fungi of the genus *Fusarium*, p. 210. 1950.

This species accounted for 4.3% of the *Fusarium* isolates obtained during this study. Of the 58 isolates obtained, three were from soil from Alberta, seven from Saskatchewan, 41 from Manitoba, three from Ontario, one from Quebec, and three from Prince Edward Island.

FUSARIUM SAMBUCINUM Fuckel var. COERULEUM Wr.

Wollenweber, Ann. Mycol. 15 : 55. 1917.
Gordon, Can. J. Botany, 30 : 227 and 230. 1952.

This variety of *F. sambucinum* constituted approximately 1% of the *Fusarium* isolates. Of the 14 isolates that were encountered, one was from British Columbia, one from Saskatchewan, 10 from Manitoba, and two from Nova Scotia.

Section Liseola Wr., Sherb., Rg., Johann, & Bailey

Wollenweber, Sherbakoff, Reinking, Johann and Bailey, J. Agr. Research, 30 : 841. 1925.
Wollenweber, Fusarium-Monographie, pp. 388–391. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 96–98. 1935.
Snyder and Hansen, Am. J. Botany, 32 : 660–661. 1945.
Raillo, Fungi of the genus *Fusarium*, pp. 257–260. 1950.

FUSARIUM MONILIFORME Sheld. emend. Snyder & Hansen

Snyder and Hansen, Am. J. Botany, 32 : 664. 1945.
Sheldon, Nebraska Agr. Expt. Sta. Ann. Rept. 17 : 23–32. 1904.
Wollenweber, Fusarium-Monographie, pp. 391–393. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 98–99. 1935.

Syn. *F. anthophilum* (A. Br.) Wr.
Raillo, Fungi of the genus *Fusarium*, p. 263. 1950.

F. anthophilum (A. Br.) Wr. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 263. 1950.

F. anthophilum (A. Br.) Wr. form 2 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 264. 1950.
F. neoceras Wr. & Rg. var. *subglutinans* (Wr. & Rg.) Raillo
Raillo, Fungi of the genus *Fusarium*, p. 263. 1950.

F. moniliforme has been reported previously to occur in Manitoba soils (1, 6). It was not found in the soil of permanent plots at Winnipeg, Man., from 1936 to 1942 (5). During the present study *F. moniliforme* was encountered rarely. Only two isolates were obtained, one from a sample of soil from Manitoba and the other from Ontario.

Section Elegans Wr.

Wollenweber, Phytopathology, 3 : 28. 1913.
Wollenweber, Fusarium-Monographie, pp. 400–406. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 104–109. 1935.
Snyder and Hansen, Am J. Botany, 27 : 64–67. 1940.
Raillo, Fungi of the genus *Fusarium*, pp. 238–239. 1950.
Gordon, Can. J. Botany, 30 : 236. 1952.

Syn. Subsection Euelegans Raillo
Raillo, Fungi of the genus *Fusarium*, p. 239. 1950.
Subsection Pseudoroseum Raillo
Raillo, Fungi of the genus *Fusarium*, p. 239. 1950.
Subsection Pseudomartiella Raillo
Raillo, Fungi of the genus *Fusarium*, p. 239. 1950.

Of the various species and varieties of *Fusarium* isolated from soil in Canada, *F. oxysporum* Schlecht. and *F. oxysporum* var. *redolens* (Wr.) Gordon, classified in this section of the genus, have been encountered most frequently (1, 5).

FUSARIUM OXYSPORUM Schlecht. emend. Snyder & Hansen
Snyder and Hansen, Am. J. Botany, 27 : 66. 1940.
Wollenweber, Fusarium-Monographie, pp. 416–418. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 117–118. 1935.
Gordon, Can. J. Botany, 30 : 236–237. 1952.

Syn. Subsection Euelegans Raillo
Raillo, Fungi of the genus *Fusarium*, p. 239. 1950.
Subsection Pseudoroseum Raillo
Raillo, Fungi of the genus *Fusarium*, p. 239. 1950.
F. conglutinans Wr. form 2 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 249. 1950.
F. oxysporum Schlecht. form 1 Raillo (non Wr.)
Raillo, Fungi of the genus *Fusarium*, p. 253. 1950.
F. oxysporum Schlecht. form 2 Raillo (non Wr.)
Raillo, Fungi of the genus *Fusarium*, p. 253. 1950.
F. oxysporum Schlecht. form 3 Raillo (non Wr.)
Raillo, Fungi of the genus *Fusarium*, p. 253. 1950.

F. oxysporum was first reported to be present in Canadian soils in 1933 (1). From 1936 to 1942, this species, including its variety *redolens*, constituted approximately 50% of isolates of *Fusarium* obtained from the soil of permanent cereal plots at Winnipeg, Man. (5).

Of the 423 isolates of *F. oxysporum* obtained from soil during the present study, 11 were obtained from British Columbia, one from Alberta, 10 from Saskatchewan, 256 from Manitoba, 56 from Ontario, 36 from Quebec, 17 from Nova Scotia, and 36 from Prince Edward Island. This species, excluding its variety *redolens*, accounted for approximately 31% of the isolates of *Fusarium*.

FUSARIUM OXYSPORUM Schlecht. emend. Snyder & Hansen var. *redolens* (Wr.) Gordon

Gordon, Can. J. Botany, 30 : 238. 1952.

Syn. Section Elegans Wr. Subsection Pseudomartiella Raillo
Raillo, Fungi of the genus *Fusarium*, p. 257. 1950.

This variety of *F. oxysporum* was reported previously to be commonly encountered in the soil of cereal plots at Winnipeg, Man. (5). During the present study, it comprised approximately 36% of the isolates of *Fusarium*. Of the 479 isolates of this variety obtained from soil, 13 were from British Columbia, 25 from Saskatchewan, 402 from Manitoba, eight from Ontario, 23 from Quebec, five from Nova Scotia, and three from Prince Edward Island. *F. oxysporum* var. *redolens* occurs frequently among isolates of fungi from the roots of cereals and other cultivated crops in Manitoba.

Section Martiella Wr.

Wollenweber, Phytopathology, 3 : 30. 1913.
Wollenweber and Reinking, Die Fusarien, pp. 127–129. 1935.
Snyder and Hansen, Am. J. Botany, 28 : 738–740. 1941.
Gordon, Can. J. Botany, 30 : 238–239. 1952.
Raillo, Fungi of the genus *Fusarium*, p. 219. 1950.

F. solani (Mart.) App. & Wr. emend. Snyder & Hansen, classified in this section of the genus, was isolated during this study.

FUSARIUM SOLANI (Mart.) App. & Wr. emend. Snyder & Hansen

Snyder and Hansen, Am. J. Botany, 28 : 740. 1941.
Wollenweber and Reinking, Die Fusarien, p. 135. 1935.
Wollenweber, Fusarium-Monographie II, pp. 181–182. 1944–45.
Snyder and Hansen, Am. J. Botany, 32 : 662. 1945.
Raillo, Fungi of the genus *Fusarium*, pp. 232–233. 1950.
Gordon, Can. J. Botany, 30 : 239–240. 1952.

Syn. *F. javanicum* Koord. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 229. 1950.

F. javanicum Koord. subsp. ensiforme (Wr. & Rg.) Raillo
Raillo, Fungi of the genus *Fusarium*, p. 229. 1950.
F. javanicum Koord. var. *radicicola* Wr. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 230. 1950.
F. martii App. & Wr. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 231. 1950.
F. martii App. & Wr. var. *minus* Sherb. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 232. 1950.
F. martii App. & Wr. var. *minus* Sherb. form 2 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 232. 1950.
F. martii App. & Wr. var. *minus* Sherb. form 3 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 232. 1950.
F. solani (Mart.) App. & Wr. form 1 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 233. 1950.
F. solani (Mart.) App. & Wr. form 2 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 233. 1950.
F. solani (Mart.) App. & Wr. form 3 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 233. 1950.
F. solani (Mart.) App. & Wr. form 4 Raillo
Raillo, Fungi of the genus *Fusarium*, p. 233. 1950.

F. solani has been isolated previously from soil in Manitoba (1, 5). During the present study it was one of the more commonly isolated species, comprising approximately 10% of the isolates of *Fusarium*. Of the 131 isolates of this species that were obtained from soil, four were from British Columbia, one from Saskatchewan, 102 from Manitoba, eight from Ontario, 13 from Quebec, and three from Prince Edward Island. *F. solani* occurs commonly among isolates of fungi obtained from the diseased basal parts of cultivated plants.

Discussion

As *Fusarium* accounted for only about 5% of the total fungus colonies that developed on the soil dilution plates during this study, representatives of this genus evidently constitute a relatively small proportion of the total fungus flora of the soils examined. There is little doubt, however, that they can account for a greater proportion of the fungus flora when soil samples are taken from particular locations. It was found previously, for example, that when samples of soil were taken from a series of permanent cereal plots at Winnipeg, Man., *Fusarium* species averaged approximately 16% of the fungus flora over a period of seven years (5). Although samples of soil examined microbiologically during the present study were taken over a wide geographic area, the identity of the majority of the species and varieties of *Fusarium* isolated was the same as from the series of cereal plots just mentioned (5).

The most striking characteristic of the *Fusarium* flora of the soil in Canada is the relatively large proportion of it that can be attributed to the presence of three species, namely, *F. oxysporum*, *F. equiseti*, and *F. solani*. During this study these species accounted for approximately 90% of the 1348 isolates of

Fusarium. *F. oxysporum*, however, was by far the most prevalent species as it represented approximately 67% of the isolates. The preponderance of this species in Canadian soils is accounted for to a large extent by the presence of its variety *redolens*, which alone represented 53% of the isolates of *F. oxysporum* and 36% of the isolates of *Fusarium*. Although *F. equiseti* and *F. solani* were not encountered nearly as frequently as *F. oxysporum* they represented approximately 13% and 10%, respectively, of the *Fusarium* flora during the present study.

From the standpoint of cereal pathology it is of interest that all of the species isolated during this study, except *F. merismoides*, have been found in Canada in the past among isolates from cereals exhibiting symptoms of common root rot and have been reported also to be seed-borne by cereals (3) and by other cultivated plants (4). The relative importance of these species in the production of disease in cereals has been previously appraised (3). It is sufficient to mention here that, of the species isolated, undoubtedly *F. culmorum* is the most important in that respect. While this species was not shown to be of common occurrence in the soil during this study, evidence has been presented previously that it may occur more commonly in a particular habitat, such as permanent cereal plots (5). The marked predominance of *F. oxysporum* in Canadian soils commands attention because of the ability of this species to cause destructive wilts of many different species of cultivated plants, but fortunately its importance in cereal disease production is negligible.

The apparent absence of certain *Fusarium* species in Canadian soils is worthy of note. Investigations of the *Fusarium* flora of the soil have so far failed to demonstrate the presence of *F. graminearum* Schwabe and *F. nivale* (Fr.) Ces., two widely recognized primary pathogens of cereals.

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THE TAXONOMY AND HABITATS OF THE FUSARIUM SPECIES IN TRINIDAD, B.W.I.[1]

By W. L. Gordon[2]

Abstract

A taxonomic study was made of isolates of *Fusarium* obtained by the writer from various habitats in Trinidad, B.W.I., during the early months of 1955. Initial phases of this study were carried out at the Imperial College of Tropical Agriculture, Trinidad, while the later phases were completed at the Plant Pathology Laboratory, Winnipeg, Man. Isolates of *Fusarium* were obtained from a total of 41 different species and botanical varieties of host plants, from scale insects, and from soil. A total of 11 species and two varieties of *Fusarium*, classified in 10 sections of the genus, were identified among the various isolates. These sections, species, and varieties are: section Macroconia, *F. coccophilum* (Desm.) Wr. & Rg.; Spicarioides, *F. decemcellulare* Brick; Pseudomicrocera, *F. coccidicola* P. Henn.; Arachnites, *F. larvarum* Fuckel; Arthrosporiella, *F. semitectum* Berk. & Rav.; Gibbosum, *F. equiseti* (Cda.) Sacc.; Lateritium, *F. lateritium* Nees, *F. stilboides* Wr.; Liseola, *F. moniliforme* Sheld., *F. moniliforme* var. *anthophilum* (A. Br.) Wr., *F. moniliforme* var. *subglutinans* Wr. & Rg.; Elegans, *F. oxysporum* Schlecht. emend. Snyder & Hansen; Martiella, *F. solani* (Mart.) App. & Wr. emend. Snyder & Hansen. Six of these species are reported for the first time to occur in Trinidad, namely, *F. coccophilum*, *F. decemcellulare*, *F. larvarum*, *F. equiseti*, *F. stilboides*, and *F. solani*. *Fusarium semitectum* and *F. moniliforme* were most frequently encountered.

The ascomycetous or perfect state of *F. coccophilum*, *Nectria ecoccophila* Wr.; of *F. decemcellulare*, *Calonectria rigidiuscula* (Berk. & Brme.) Sacc.; of *F. moniliforme*, *Gibberella fujikuroi* (Saw.) Wr.; and of *F. solani*, *Hypomyces solani* (Rke. & Berth.) emend. Snyder & Hansen were collected in nature.

This paper is illustrated with drawings of the majority of the *Fusarium* species and the ascomycetous states mentioned.

Introduction

This study was begun in the early part of 1955, when the writer spent approximately four months in Trinidad collecting and culturing specimens of *Fusarium* encountered there in various habitats. These initial phases of the study were centered in the Department of Plant Pathology, the Imperial College of Tropical Agriculture, St. Augustine, where the Principal, Dr. G. A. C. Herklots, made available laboratory space and other facilities required for the microscopic examination and culture of specimens. Later phases of this work were completed on return to the Laboratory of Plant Pathology, Science Service, Winnipeg, Man.

Materials and Methods

Sources of Isolates

The isolates of *Fusarium* involved in this taxonomic study were obtained chiefly from specimens collected on the premises of the Imperial College of Tropical Agriculture (I.C.T.A.) and in the adjacent vicinity of St. Augustine. Series of plots located at I.C.T.A. containing a wide range of named species

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and varieties of tropical and other plants were the most fruitful source of specimens. At the time they were examined many of these plants were approaching, or had reached, maturity. It was thus possible to obtain seeds directly from some of them for plating tests to determine whether they were harboring *Fusarium*. The scale insects that were commonly encountered, particularly on the leaves of *Citrus* spp., were another source of isolates. Diligent examination of the older leaves on the trees with the aid of a hand lens usually resulted in the detection of occasional scale insects bearing sporodochia of *Fusarium* species.

In addition to the isolates obtained from the sources just mentioned, several others were acquired from a qualitative, microbiological examination of samples of soil. Some of these samples were taken from the plots at I.C.T.A., others from different localities in Trinidad and Tobago. The local origin of each sample examined that yielded *Fusarium* is given below.

Methods of Obtaining Isolates

Isolates of *Fusarium* were obtained from the specimens collected in nature by one or other of three methods: monospore culture, mass culture, and dilution plate techniques. When the plant or insect bore conidia or perithecia of the ascomycetous state of *Fusarium*, the monospore culture technique was employed directly in obtaining monoconidial or single ascospore cultures. The monospore culture technique consisted of making a dilute suspension of conidia or ascospores in acidified, sterile water, and pouring part of the suspension over the surface of previously poured plain agar plates. After the excess water was decanted off the surface of the agar, the plates were inverted, and kept at room temperature for 16 to 20 hr. to allow germination of the conidia or ascospores to take place. The surface of the agar was then examined under a dissecting microscope (35×) for germinating conidia or ascospores. When encountered, these were picked up singly along with a small piece of agar with the aid of a fine, curve-pointed needle and transferred to separate slants of potato-sucrose agar (2% sucrose, 4% agar).

Specimens, such as samples of seed and discolored or decayed plant parts, which did not bear conidia on their surface were subjected to the usual mass culture technique. Before specimens of this type were plated out on potato-sucrose agar, they were surface-disinfected with a 1:1000 mercuric chloride solution followed by repeated rinsing in sterile water. If *Fusarium* was present in the specimens plated, colonies bearing conidia were usually present on the isolation plates in three to four days. Some of the conidia were then removed from each type of colony for immediate monospore culture.

Samples of soil were examined for *Fusarium* by the customary dilution plate technique. The samples were relatively small, consisting of about half a pint of soil, but were sufficient in size for a qualitative examination. Samples were usually air-dried for 24 hr. after their collection. An aliquot of 5 gm. of soil was then taken from each and diluted in sterile water to

1:200. One milliliter of each soil dilution was then transferred to each of four Petri dishes to which was then added 10 cc. of acidified potato-sucrose agar (approximately pH 4). These Petri dish cultures were incubated for four to six days at room temperature, and then examined for colonies of *Fusarium*. At dilution of 1:200, 30 to 60 colonies of fungi usually appeared on each and this number facilitated rapid detection of *Fusarium* colonies. From each of the colonies of *Fusarium* that developed on the dilution plates a sample of conidia was taken for monospore culture.

Although different methods of isolation were employed in this study, single spore isolates were obtained in culture within a few days of the time each specimen was collected in nature. These single spore isolates can therefore be regarded as the wild types of the species and varieties found in nature. This taxonomic study of the species and varieties of *Fusarium* isolated in Trinidad is based on these single spore isolates of the wild types.

Drawings

Drawings were made with the aid of a camera lucida.

Classification of the Isolates

The system of classification and nomenclature presented by Wollenweber and Reinking (10) in 1935 was in general followed in classifying the isolates of *Fusarium*. It was modified in several respects, however, in line with the concept of certain sections and species as proposed by Wollenweber (9), Snyder and Hansen (5, 6, 7), and Gordon (2, 3).

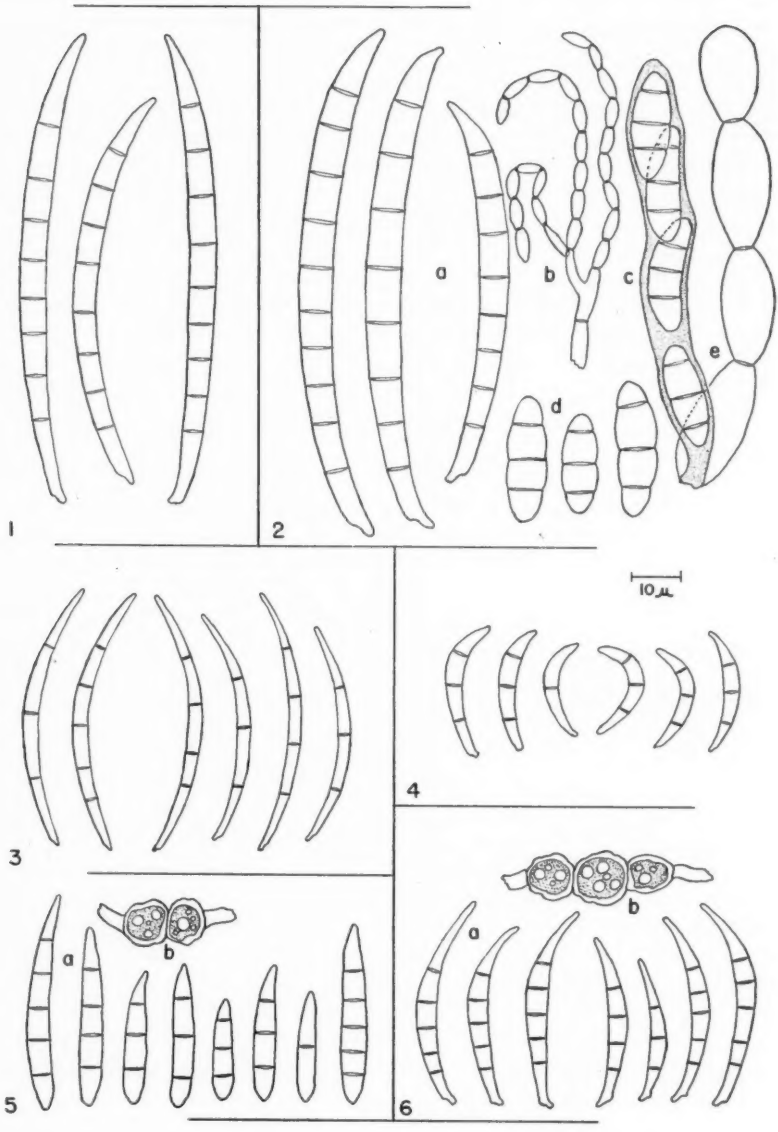
According to the method of classification adopted by the writer, which is set forth in the taxonomic section of this paper, a total of 11 species and two varieties of *Fusarium* were identified among the isolates obtained from various habitats. These species and varieties represented 10 sections of the genus, namely, Macroconia, Spicarioides, Pseudomicrocera, Arachnites, Arthrosporiella, Gibbosum, Lateritium, Liseola, Elegans, and Martiella.

As the complete synonymy of the species and varieties of *Fusarium* included in this paper is exceedingly voluminous, particularly since the publication of "Grebi roda Fusarium" by Raillo (4) in 1950, only those synonyms that are likely to be encountered in recent scientific papers and abstracting journals have been given. A more complete synonymy may be found in Fusarium-Monographie by Wollenweber (8, 9), in Die Fusarien by Wollenweber and Reinking (10), and in the publication by Raillo (4).

Section Macroconia Wr.

Wollenweber and Reinking, Die Fusarien, pp. 27–29. 1935.
Wollenweber, Fusarium-Monographie II, pp. 112–113. 1944–45.

One species, namely, *Fusarium coccophilum* (Desm.) Wr. & Rg., classified in this section of the genus was identified among the collections obtained from scale insects.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FUSARIUM COCCOPHILUM (Desm.) Wr. & Rg. (Fig. 1). Die Fusarien, p. 34. 1935.—Doidge, Bothalia, 3(3): 335–337. 1938.—Wollenweber, Fusarium-Monographie II, pp. 117–118. 1944–45.

F. episphaeria (Tode) Snyder & Hansen f. *coccophila* (Desm.) Snyder & Hansen. Am. J. Botany, 32 : 662. 1945.

Habitats: On scale insects on the leaves of *Citrus sinensis* Osbeck (sweet orange) and *Citrus maxima* Merr. (*C. paradisi* hort.) (grapefruit).

Conidia taken from well-developed sporodochia of *F. coccophilum* on scale insects present on the leaves of sweet orange and grapefruit consistently failed to germinate so that this species was not established in culture. The sporodochia may have developed during the wet season several months previous to the time of their collection.

The ascomycetous or perfect state of *Fusarium coccophilum* is *Nectria ecoccophila* Wr. (9).

NECTRIA ECOCCOPHILA Wr. Fusarium-Monographie II, pp. 118–119. 1944–45.

Sphaerostilbe coccophila Tul. Carpol. Vol. 3; p. 105. 1865.

Sphaerostilbe flammea Tul. Carpol. Vol. 3; p. 104. 1865.

Nectria coccophila (Tul.) Wr. & Rg. Die Fusarien, pp. 34–46. 1935.

Nectria episphaeria (Tode) Snyder & Hansen f. *coccophila* (Desm.) Snyder & Hansen. Am. J. Botany, 32 : 662. 1945.

Habitat: On a scale insect on a leaf of *Citrus sinensis* Osbeck (sweet orange) along with the imperfect state (sporodochia) of *F. coccophilum*. A single group of four perithecia was found. Ascospores failed to germinate. *Nectria ecoccophila* has previously been reported to occur in Trinidad under the names *Sphaerostilbe coccophila* Tul. and *Sphaerostilbe flammea* Tul. (1).

Section Spicarioides Wr., Sherb., Rg., Johan., & Bailey

Wollenweber, Sherbakoff, Reinking, Johann, and Bailey, J. Agr. Research, 30 : 841. 1925.

Wollenweber, Fusarium-Monographie, pp. 310–311. 1931.

Wollenweber and Reinking, Die Fusarien, p. 36. 1935.

The only species classified in this section of the genus, namely, *Fusarium decemcellulare* Brick, was encountered occasionally.

FIG. 1. Section Macroconia, *F. coccophilum;* conidia.

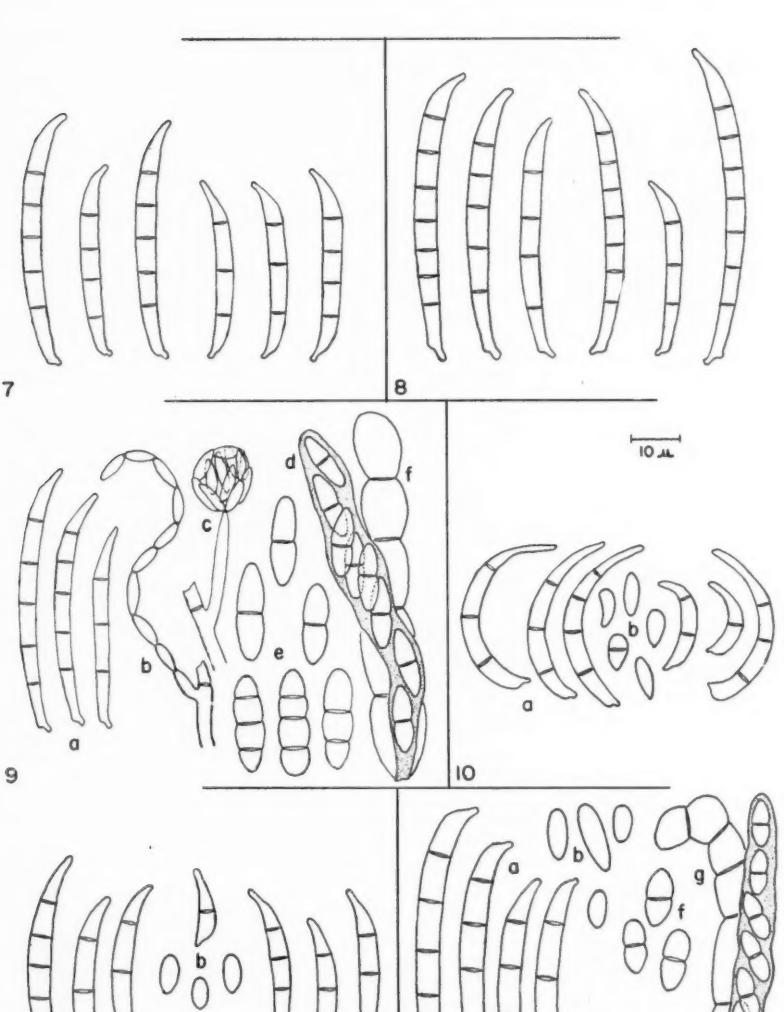
FIG. 2. Section Spicarioides, *Calonectria rigidiuscula* (*F. decemcellulare*): *a*, macroconidia; *b*, microconidia in chains; *c*, ascus with four ascospores; *d*, mature ascospores; *e*, a paraphysis.

FIG. 3. Section Pseudomicrocera, *F. coccidicola:* conidia.

FIG. 4. Section Arachnites, *F. larvarum:* conidia.

FIG. 5. Section Arthrosporiella, *F. semitectum: a*, conidia; *b*, intercalary chlamydospores.

FIG. 6. Section Gibbosum, *F. equiseti: a*, conidia; *b*, intercalary chlamydospores.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FUSARIUM DECEMCELLULARE Brick (Fig. 2, *a*, *b*). Jber. Ver. Angew. Botanik, 6: 227. 1908.—Wollenweber, Fusarium-Monographie, pp. 311–312. 1931. —Wollenweber and Reinking, Die Fusarien, p. 37. 1935.

Habitats: Fruiting through fissures in the bark of dead branches of *Theobroma cacao* L. (cocoa) and present on dead branches of *Gossypium purpurascens* Poir (perennial cotton).

The ascomycetous or perfect state of *F. decemcellulare* is:

CALONECTRIA RIGIDIUSCULA (Berk. & Br.) Sacc. (Fig. 2, *c*, *d*, *e*). Wollenweber, Fusarium-Monographie, pp. 312–314. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 37–38. 1935.

Scoleconectria tetraspora Seaver. N. Amer. Flora, 3 : 37. 1910.

Calonectria tetraspora (Seav.) Sacc. & Trott. Syll. Fungorum, 22 : 487. 1913.

Habitats: The same as for the imperfect state, *F. decemcellulare*.

The occurrence of *Calonectria rigidiuscula* in Trinidad has previously been reported as *Scoleconectria tetraspora* Seaver. The perithecia were found on cocoa pods (1).

Section Pseudomicrocera (Petch) Wr.

Wollenweber and Reinking, Die Fusarien, p. 40. 1935.

Wollenweber, Fusarium-Monographie II, p. 123. 1944–45.

This section of the genus was represented by one species, namely, *Fusarium coccidicola* P. Henn.

FUSARIUM COCCIDICOLA P. Henn. (Fig. 3). Wollenweber, Fusarium-Monographie II, pp. 123–124. 1944–45.

Pseudomicrocera henningsii (Koord.) Petch. Trans. Brit. Mycol. Soc. 7 : 164. 1921.

Fusarium juruanum P. Henn. Wollenweber and Reinking, Die Fusarien, p. 41. 1935.

Habitats: On scale insects on the leaves of *Citrus aurantifolia* Swingle (lime); *C. nobilis* Lour. var. *deliciosa* Swingle (tangerine); *C. maxima* Merr. (*C. paradisi* hort.) (grapefruit); *C. sinensis* Osbeck (sweet orange); *Mangifera indica* L. (mango).

FIG. 7. Section Lateritium, *F. lateritium:* conidia.

FIG. 8. Section Lateritium, *F. stilboides:* conidia.

FIG. 9. Section Liseola, *Gibberella fujikuroi* (*F. moniliforme*): *a*, macroconidia; *b*, microconidia in chains; *c*, microconidia in a false head; *d*, ascus with eight ascospores; *e*, mature ascospores; *f*, a paraphysis.

FIG. 10. Section Liseola, *F. moniliforme* var. *anthophilum: a*, macroconidia; *b*, microconidia.

FIG. 11. Section Elegans, *F. oxysporum: a*, macroconidia; *b*, microconidia; *c*, terminal chlamydospore; *d*, intercalary chlamydospore.

FIG. 12. Section Martiella, *Hypomyces solani* (*F. solani*): *a*, macroconidia; *b*, microconidia; *c*, terminal chlamydospore; *d*, intercalary chlamydospore; *e*, ascus with eight ascospores; *f*, mature ascospores; *g*, a paraphysis.

This species of *Fusarium*, reported as *Pseudomicrocera henningsii* (Koord.) Petch, was known previously to occur in Trinidad on scale insects on *Citrus* and on a rose stem (1). The ascomycetous or perfect state of *F. coccidicola* has been reported to be *Calonectria diploa* (Berk. & Curt.) Wr. by Wollenweber and Reinking (10).

Section Arachnites Wr.

Wollenweber, Ann. Mycol. 15 : 2. 1917.
Wollenweber, Fusarium-Monographie, p. 314. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 42–43. 1935.

Fusarium larvarum Fuckel was the only species isolated that belonged in this section of the genus.

FUSARIUM LARVARUM Fuckel (Fig. 4). Wollenweber and Reinking, Die Fusarien, pp. 43–44. 1935.

Fusarium aspidioti Sawada. Wollenweber, Fusarium-Monographie, pp. 316–317. 1931.

F. nivale (Fr.) Ces. emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 662. 1945.

Habitats: On scale insects on the leaves of *Citrus sinensis* Osbeck (sweet orange), and *Citrus maxima* Merr. (*C. paradisi* hort.) (grapefruit).

This species was less frequently encountered on scale insects on *Citrus* spp. than *F. coccidicola* and *F. coccophilum*. An ascomycetous or perfect state of *F. larvarum* has not yet been reported.

Section Arthrosporiella Sherb.

Sherbakoff, N.Y. Cornell Agr. Expt. Sta. Memoir, 6 : 161. 1915.
Wollenweber, Fusarium-Monographie, pp. 322–324. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 57–58. 1935.

Fusarium semitectum Berk. & Rav., classified in this section of the genus, was one of the most frequently encountered species in nature.

FUSARIUM SEMITECTUM Berk. & Rav. (Fig. 5). Grevillea, 3 : 98. 1875.—Wollenweber, Fusarium-Monographie, pp. 324–325. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 58–59. 1935.—Gordon, Can. J. Botany, 30 : 222–223. 1952.

F. semitectum Berk. & Rav. var. *majus* Wr. Fusarium-Monographie, pp. 325–327. 1931.—Wollenweber and Reinking, Die Fusarien, p. 59. 1935.

F. diversisporum Sherb. N.Y. Cornell Agr. Expt. Sta. Memoir, 6 : 161–166. 1915.—Wollenweber, Fusarium-Monographie, pp. 327–328. 1931.—Wollenweber and Reinking, Die Fusarien, p. 61. 1935.

F. roseum Lk. emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 663. 1945.

Habitats: *Clitoria ternatea* L. (blue and white pea). Isolated from seed.
Coix lacryma-jobi L. (dwarf adlay). Present on and in the seed.
Corchorus olitorius L. (nalta jute). Fruiting on ripe seed capsules.
Crotalaria juncea L. (sunn hemp). Isolated from seed.
Eleusine indica L. (goose grass). Fruiting on surface of seed.
Glycine max Merr. (soybean). Isolated from discolored basal parts of a plant.
Helianthus tuberosus L. (girasol). Fruiting on mature stems.
Hibiscus esculentus L. (okra). Fruiting on the surface of seed in ripe pods.
Luffa acutangula Roxb. (Chinese okra). Isolated from seed.
Musa paradisiaca L. var. *sapientum* Kuntze (banana). Fruiting on the surface of a discolored peduncle.
Sansevieria zeylanica Willd. (bowstring hemp). Fruiting on the surface of a marginal leaf blight.
Soil. Isolated from soil taken from a vegetable garden in the vicinity of San Juan and from a sugar cane plot, I.C.T.A.
Solanum melongena L. var. *esculentum* Nees (melongene). Fruiting on mature peduncles.
Sorghum vulgare Pers. (sorghum). Fruiting abundantly on the mature inflorescence and seed of the varieties Atlas and Caprock.
Vigna sinensis Endl. (cowpea). Isolated from seed.

Section Gibbosum Wr.

Wollenweber and Reinking, Die Fusarien, pp. 61–62. 1935.
Snyder and Hansen, Am. J. Botany, 32 : 663. 1945.
Gordon, Can. J. Botany, 30 : 223–224. 1952.

FUSARIUM EQUISETI (Cda.) Sacc. Syll. Fungorum 4 : 707–708. 1886.—Wollenweber and Reinking, Die Fusarien, pp. 63–64. 1935.—Gordon, Can. J. Botany, 30 : 225. 1952.

F. scirpi Lamb. & Fautr. Wollenweber and Reinking, Die Fusarien, p. 66. 1935.—Gordon, Can. J. Botany, 30 : 223–225. 1952.

F. roseum Lk. emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 663. 1945.

Habitat: A single isolate of *F. equiseti* was obtained from grassland soil in the vicinity of Scarborough, Tobago.

Section Discolor Wr.

Wollenweber and Reinking, Die Fusarien, pp. 69–72. 1935.

Isolates of *Fusarium* belonging in this section of the genus were not obtained during this study. Only one species, namely, *F. sambucinum* Fuckel, of the section Discolor, is known to occur in Trinidad. It was reported from banana material (1).

Section Lateritium Wr.

Wollenweber and Reinking, Fusarium-Monographie, pp. 368–370. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 86–88. 1935.
Snyder and Hansen, Am. J. Botany, 32 : 664. 1945.

This section of the genus was represented among the isolates of *Fusarium* by *F. lateritium* Nees and *F. stilboides* Wr.

FUSARIUM LATERITIUM Nees (Fig. 7). Wollenweber, Fusarium-Monographie, pp. 370–373. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 88–90. 1935.

F. sarcochroum (Desm.) Sacc. Wollenweber and Reinking, Die Fusarien, pp. 95–96. 1935.

F. lateritium Nees emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 664. 1945.

Habitats: Isolated from a dieback of twigs of *Citrus maxima* Merr. (*C. paradisi* hort.) (grapefruit); from scale insects on the leaves of *Citrus sinensis* Osbeck (sweet orange) and on the leaves of *Citrus maxima* Merr. (*C. paradisi* hort.) (grapefruit).

FUSARIUM STILBOIDES Wr. Fusarium-Monographie, p. 385. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 94–95. 1935.

F. lateritium Nees emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 664 1945.

Habitat: Isolated from a commonly occurring decay of the immature berries of *Coffea liberica* Hiern (Liberian coffee).

Section Liseola Wr., Sherb., Rg., Johan., & Bailey

Wollenweber, Sherbakoff, Reinking, Johann, and Bailey, J. Agr. Research, 30 : 841. 1925.
Wollenweber and Reinking, Die Fusarien, pp. 96–98. 1935.
Snyder and Hansen, Am. J. Botany, 32 : 664. 1945.

This section of the genus was represented among the isolates by *F. moniliforme* Sheld. and two varieties of this species, namely, var. *subglutinans* Wr. & Rg. and var. *anthophilum* (A. Br.) Wr.

FUSARIUM MONILIFORME Sheld. Nebraska Agr. Expt. Sta. Ann. Rept. 17 : 23–32. 1904.—Wollenweber and Reinking, Die Fusarien, pp. 98–99. 1935.

F. moniliforme Sheld. var. *minus* Wr. Fusarium-Monographie, p. 397. 1931.—Wollenweber and Reinking, Die Fusarien, p. 102. 1935.

F. lactis Pir. & Rib. Wollenweber and Reinking, Die Fusarien, p. 103. 1935.

F. moniliforme Sheld. emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 664. 1945.

Habitats: *Andropogon gayanus* Kunth. Isolated from seed.

Corchorus capsularis L. (jute). Isolated from seed.

Crotalaria usaramoensis E. G. Baker. Isolated from basal parts of plants with foot root.

Crotalaria valetonii Backer. Isolated from basal parts of a plant with foot rot.

Hibiscus esculentus L. (okra). Fruiting on the surface of seeds in ripe pods.

Maranta arundinacea L. (arrowroot). Isolated from discolored basal parts of a plant.

Musa paradisiaca L. var. *sapientum* Kuntze (banana). Isolated from a discolored leaf sheath.

Ricinus communis L. (castor oil plant). Isolated from seed.

Rottboellia exaltata L. (corn grass). Fruiting on discolored nodes of plants.

Soil. Isolated from a sample of forest soil, Long Stretch.

Sorghum vulgare L. (sorghum). Fruiting on inflorescence and seed of the varieties Alpha and Atlas.

Theobroma cacao L. (cocoa). Fruiting on the surface of a dead branch.

Zea mays L. Isolated from the seed.

The ascomycetous or perfect state of *F. moniliforme* is *Gibberella fujikuroi* (Saw.) Wr. Fusarium-Monographie, p. 514. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 99–100. 1935.

Lisea fujikuroi Saw. Ber. naturhist. Ges. Formosa, 31 : 131–133. 1917.

Gibberella moniliformis (Sheld.) Wineland. J. Agr. Research, 28 : 920. 1924.—Wollenweber, Fusarium-Monographie, pp. 393–395. 1931.

G. moniliforme (Sheld.) Snyder & Hansen pr. p. Am. J. Botany, 32 : 664. 1945.

Habitat: On the surface of seeds in ripe pods of *Hibiscus esculentus* L. (okra) along with the conidial stage, *F. moniliforme*.

FUSARIUM MONILIFORME Sheld. var. SUBGLUTINANS Wr. & Rg. Fusarium-Monographie, p. 397. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 100–101. 1935.

F. neoceras Wr. & Rg. Fusarium-Monographie, p. 399. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 103–104. 1935.

F. moniliforme Sheld. emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 664. 1945.

Habitats: *Citrus maxima* Merr. (*C. paradisi* hort.) (grapefruit). Isolated from a dieback of twigs of grapefruit.

Hibiscus sabdariffa L. (roselle). Fruiting on the surface of mature pods.

Saccarum officinarum L. (sugar cane). Isolated from a reddish discoloration of nodal tissue.

Soil. Isolated from a sample of soil from the Blanchisseuse Road area, Northern Range.

Urena lobata L. (aramina fiber). Isolated from seed.

FUSARIUM MONILIFORME Sheld. var. ANTHOPHILUM (A. Br.) Wr. Fusarium-Monographie, pp. 397–399. 1931.—Wollenweber and Reinking, Die Fusarien, pp. 101–102. 1935.

F. succisae (Schroet.) Sacc. Syll. Fungorum, 10 : 724. 1892.

F. moniliforme Sheld. emend. Snyder & Hansen pr. p. Am. J. Botany, 32 : 664. 1945.

Habitat: *Cocos nucifera* L. (coconut). Isolated from the ripe husk of a coconut.

F. moniliforme var. *anthophilum* was known previously to occur in Trinidad. A culture of a *Fusarium* (I.M.I. 43499) isolated from copra in Trinidad and forwarded by the Commonwealth Mycological Institute to the writer in 1951 for identification proved to be this fungus. This variety of *F. moniliforme* has also been reported, as *Fusarium succisae* Schroet. (1), to occur in Trinidad on banana material.

Section Elegans Wr.

Wollenweber, Fusarium-Monographie, pp. 400–406. 1931.
Wollenweber and Reinking, Die Fusarien, pp. 104–109. 1935.
Snyder and Hansen, Am. J. Botany, 27 : 64–67. 1940.
Gordon, Can. J. Botany, 30 : 236. 1952.

Isolates belonging in this section of the genus are included in *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen.

FUSARIUM OXYSPORUM Schlecht. emend. Snyder & Hansen (Fig. 11). Am. J. Botany, 27 : 66. 1940.—Wollenweber and Reinking, Die Fusarien, pp. 117–118. 1935.—Gordon, Can. J. Botany, 30 : 236–238. 1952.

F. angustum Sherb. Wollenweber and Reinking, Die Fusarien, p. 113. 1935.

F. bostrycoides Wr. & Rg. Die Fusarien, pp. 109–110. 1935.

F. bulbigenum Cke. & Mass. Wollenweber and Reinking, Die Fusarien, pp. 113–114. 1935.

F. conglutinans var. *citrinum* Wr. Wollenweber and Reinking, Die Fusarien, pp. 111. 1935.

F. orthoceras App. & Wr. Wollenweber and Reinking, Die Fusarien, pp. 111–112. 1935.

F. orthoceras App. & Wr. var. *longius* (Sherb.) Wr. Wollenweber and Reinking, Die Fusarien, pp. 112–113. 1935.

F. oxysporum Schlecht. var. *meniscoideum* Bugn. Encycl. Mycol. XI : 111–114. 1939.

F. vasinfectum Atk. f. 2. Wr. & Rg. Die Fusarien, p. 125. 1935.

F. vasinfectum Atk. var. *lutulatum* (Sherb.) Wr. Wollenweber and Reinking, Die Fusarien, p. 125. 1935.

F. vasinfectum Atk. var. *zonatum* (Sherb.) Wr. Wollenweber and Reinking, Die Fusarien, pp. 125–126. 1935.

Habitats: *Antirrhinum majus* L. (snapdragon). Isolated from basal parts of wilted plants along with a *Pythium* sp. and nematodes.

Dahlia pinnata Cav. (dahlia). Isolated from basal parts of a wilted plant along with nematodes.

Crotalaria juncea L. (sunn hemp). Isolated from the basal parts of plants with foot root.

Desmodium sp. Isolated from internally discolored basal parts of plants.

Dracaena fragrans Ker. (white rayo). Fruiting on, and isolated from, a marginal leaf blight.

Hibiscus esculentus L. (okra). Isolated from the basal parts of plants with foot rot.

Nicotiana tabacum L. (tobacco). Isolated from basal parts of a wilted tobacco plant along with *Sclerotium rolfsii* Sacc. and nematodes.

Soil. Isolated from wooded soils in the vicinity of St. Augustine, Talparo, Little Toby Road, Blanchisseuse Road, Trinidad; and Mt. Irvine Road, Tobago.

Isolated from a "good cocoa soil", Central Range.

Isolated from cultivated soil, Caparo Valley.

Isolated from soil taken from a plot of bananas, of sugar cane, and of *Colocasia esculenta* Schott. at I.C.T.A.

Vigna sinensis Savi (cowpea). Isolated from the roots and stem bases of the variety America Red.

F. oxysporum was previously known to occur in Trinidad as the cause of internal reddening of *Citrus* fruit. In addition, the occurrence of three "formae" of this species, namely, f. *conglutinans* (Wr.) Snyder & Hansen, the cause of cabbage yellows; f. *lycopersici* (Sacc.) Snyder & Hansen, the cause of tomato wilt; and f. *cubense* (E.F. Sm.) Snyder & Hansen, the cause of the destructive Panama disease of bananas, has been recorded there (1).

Section Martiella Wr.

Wollenweber and Reinking, Die Fusarien, pp. 127–129. 1935.
Snyder and Hansen, Am. J. Botany, 28 : 738–740. 1941.
Gordon, Can. J. Botany, 30 : 238–239. 1952.

Isolates belonging in this section of the genus are included in:

FUSARIUM SOLANI (Mart.) App. & Wr. emend. Snyder & Hansen (Fig. 12, *a*, *b*, *c*, *d*). Wollenweber and Reinking, Die Fusarien, p. 135. 1935.—Snyder and Hansen, Am. J. Botany, 28 : 740. 1941.—Snyder and Hansen, Am. J. Botany, 32 : 662. 1945.—Gordon, Can. J. Botany, 30 : 238–240; 245. 1952.

F. argillaceum (Fr.) Sacc. Wollenweber and Reinking, Die Fusarien, pp. 140–141. 1935.—Snyder and Hansen, Am. J. Botany, 32 : 662. 1945.

F. javanicum Koord. Wollenweber and Reinking, Die Fusarien, pp. 131–132. 1935.—Wollenweber, Fusarium-Monographie II, p. 178. 1944–45.

F. javanicum Koord. var. *ensiforme* (Wr. & Rg.) Wr. Wollenweber and Reinking, Die Fusarien, pp. 132–133. 1935.—Wollenweber, Fusarium-Monographie II, p. 180. 1944–45.

F. solani (Mart.) App. & Wr. var. *eumartii* (Carp.) Wr. pr. p. Wollenweber and Reinking, Die Fusarien, p. 138. 1935.—Wollenweber, Fusarium-Monographie II, pp. 184–185. 1944–45.

F. solani (Mart.) var. *martii* (App. & Wr.) Wr. Wollenweber and Reinking, Die Fusarien, pp. 136–137. 1935.

F. solani (Mart.) App. & Wr. var. *minus* Wr. Wollenweber and Reinking, Die Fusarien, p. 134. 1935.—Wollenweber, Fusarium-Monographie II, p. 186. 1944–45.

F. solani (Mart.) App. & Wr. var. *striatum* (Sherb.) Wr. Wollenweber and Reinking, Die Fusarien, pp. 135–136. 1935.—Wollenweber, Fusarium-Monographie II, p. 187. 1944–45.

F. solani (Mart.) var. *martii* (App. & Wr.) Wr. f. 1. Wr. Wollenweber and Reinking, Die Fusarien, p. 137. 1935.—Wollenweber, Fusarium-Monographie II, p. 183. 1944–45.

Habitats: *Corchorus capsularis* L. (jute). Isolated from the basal parts of plants with foot rot.

Crotalaria usaramoensis E. G. Baker. Isolated from the basal parts of plants with foot rot.

Crotalaria valetonii Backer. Isolated from the basal parts of plants with foot rot.

Dahlia pinnata Cav. (dahlia). Isolated from the basal parts of a wilted plant along with nematodes.

Desmodium sp. Isolated from internally discolored basal parts of a plant.

Hibiscus sabdariffa L. (roselle). Isolated from the basal parts of plants with foot rot.

Ricinus communis L. (castor oil plant). Isolated from seed.

Soil. Isolated from a "good cocoa soil", Central Range.

Isolated from a vegetable garden area, San Juan.

Isolated from a plot of *Colocasia esculenta* Schott, I.C.T.A.

Isolated from two samples of forest soil, Brigand Hill.

Isolated from grassland, along Agricultural Buildings Rd., Tobago.

Zinnia elegans Jacq. (zinnia). Isolated from the basal parts of plants with foot rot.

The ascomycetous or perfect state of *F. solani* is:

HYPOMYCES SOLANI Rke. & Berth. emend. Snyder & Hansen (Fig. 12, *e*, *f*, *g*). Wollenweber, Fusarium-Monographie, p. 427. 1931—Wollenweber and Reinking, Die Fusarien, p. 141. 1935.—Snyder and Hansen, Am. J. Botany, 28 : 741. 1941.

Hypomyces haematococcus (Berk. & Br.) Wr. Wollenweber and Reinking, pp. 139–140. 1935.

H. haematococcus (Berk. & Br.) var. *breviconus* Wr. Wollenweber and Reinking, Die Fusarien, pp. 134–135. 1935.

H. haematococcus (Berk. & Br.) var. *cancri* (Rutg.) Wr. Wollenweber and Reinking, Die Fusarien, p. 136. 1935.

H. ipomoeae (Hals.) Wr. Wollenweber and Reinking, Die Fusarien, p. 132. 1935.

H. ipomoeae (Hals.) var. *major* Wr. Wollenweber and Reinking, Die Fusarien, p. 133. 1935.

H. ipomoeae (Hals.) f. 1. Wr. Wollenweber and Reinking, Die Fusarien, p. 131. 1935.

Habitats: Perithecia of *Hypomyces solani* were collected on dead stems of *Crotalaria valetonii* Backer, of *Desmodium* sp., and of *Gossypium purpurascens* Poir (perennial cotton); and on dead inflorescences of *Gossypium barbadense* L. (sea island cotton). In each of these instances the part of the plants bearing perithecia had been in contact with the soil for some time.

Excluded. *Fusarium album* Sacc. included in a list of *Fusarium* species found in Trinidad (1) is now considered to be *Cylindrocarpon album* (Sacc.) Wr. (10).

Distribution of Species by Habitats

Andropogon gayanus Kunth
F. moniliforme

Antirrhinum majus L. (snapdragon)
F. oxysporum

Citrus aurantifolia Swingle (lime)
F. coccidicola

Citrus maxima Merr. (*C. paradisi* hort.) (grapefruit)
F. coccophilum, *F. coccidicola*, *F. larvarum*, *F. lateritium*, *F. moniliforme* var. *subglutinans*

Citrus sinensis Osbeck (sweet orange)
F. coccophilum and *Nectria ecoccophila*; *F. coccidicola*, *F. larvarum*, *F. lateritium*

Citrus nobilis Lour. var. *deliciosa* Swingle (tangerine)
F. coccidicola

Clitoria ternatea L. (white and blue pea)
F. semitectum

Cocos nucifera L. (coconut)
F. moniliforme var. *anthophilum*

Coffea liberica Hiern (Liberian coffee)
F. stilboides

Coix lacryma-jobi L. (dwarf adlay)
F. semitectum

Corchorus olitorius L. (nalta jute)
F. semitectum

Corchorus capsularis L. (jute)
F. moniliforme, *F. solani*

Crotalaria juncea L. (sunn hemp)
F. semitectum; *F. oxysporum*

Crotalaria usaramoensis E. G. Baker
F. moniliforme, *F. solani*

Crotalaria valetonii Backer
F. moniliforme; *F. solani* and *Hypomyces solani*

Dahlia pinnata Cav. (dahlia)
F. oxysporum, *F. solani*

Desmodium sp.
F. oxysporum; *F. solani* and *Hypomyces solani*

Dracaena fragrans Ker. (white rayo)
F. oxysporum

Eleusine indica (L). Gaertn. (goose grass)
F. semitectum
Glycine max Merr. (soybean)
F. semitectum
Gossypium barbadense L. (sea island cotton)
F. solani and *Hypomyces solani*
Gossypium purpurascens Poir (perennial cotton)
F. decemcellulare and *Calonectria rigidiuscula*
F. solani and *Hypomyces solani*
Helianthus tuberosus L. (girasol)
F. semitectum
Hibiscus esculentus L. (okra)
F. semitectum; F. moniliforme and *Gibberella fujikuroi; F. oxysporum*
Hibiscus sabdariffa L. (roselle)
F. moniliforme var. *subglutinans, F. solani*
Luffa acutangula Roxb. (Chinese okra)
F. semitectum
Mangifera indica L. (mango)
F. coccidicola
Maranta arundinacea L. (arrowroot)
F. moniliforme
Musa paradisiaca L. var. *sapientum* Kuntze (banana)
F. semitectum, F. moniliforme
Nicotiana tabacum L. (tobacco)
F. oxysporum; F. solani
Ricinus communis L. (castor oil plant)
F. moniliforme, F. solani
Rottboellia exaltata L. (corn grass)
F. moniliforme
Saccharum officinarum L. (sugar cane)
F. moniliforme var. *subglutinans*
Sansevieria zeylanica Willd. (bowstring hemp)
F. semitectum
Solanum melongena L. var. *esculentum* Nees (melongene)
F. semitectum
Sorghum vulgare Pers.
F. semitectum, F. moniliforme
Theobroma cacao L. (cocoa)
F. decemcellulare and *Calonectria rigidiuscula; F. moniliforme*
Urena lobata (L.) Gaertn. (aramina fiber)
F. moniliforme var. *subglutinans*
Vigna sinensis Savi (cowpea)
F. semitectum; F. oxysporum
Zea mays L. (corn)
F. moniliforme
Zinnia elegans Jacq. (zinnia)
F. oxysporum, F. solani
Scale insects (unidentified)
F. coccophilum and *Nectria ecoccophila; F. coccidicola, F. lateritium, F. larvarum*
Soil
F. semitectum, F. equiseti, F. moniliforme, F. oxysporum, F. solani

Discussion

From the standpoint of their probable economic importance the various species of *Fusarium* known to occur in Trinidad can be arranged in two groups. The first group includes those which can be regarded as being beneficial because they parasitize scale insects that occur commonly on the leaves of *Citrus* spp. These species are *F. coccophilum*, *F. coccidicola*, *F. larvarum*, and possibly *F. lateritium* although the occurrence of this latter species in nature is not confined to scale insects. While the observations on the prevalence of these species on scale insects, made during the dry season, did not suggest that they were a major factor in the control of these insects, it

is possible they might be more prevalent during the wet season. Environmental conditions would then be more favorable for infections of epidemic proportions. Further observations on the prevalence of these species are needed before their effectiveness in the biological control of scale insects can be accurately evaluated.

The second group includes those species which are known to be detrimental to plants or are usually suspected of being so because of their frequent association with disease in them. Of the species included in this group, *F. oxysporum* is undoubtedly the most important from the standpoint of disease production. Different "formae" of this species are known to cause vascular wilts, such as *F. oxysporum* f. *cubense*, the cause of Panama disease of bananas which is common and generally widespread throughout Trinidad (1). In addition, *F. oxysporum* f. *lycopersici* and *F. oxysporum* f. *conglutinans* make sporadic attacks on tomatoes and cabbage, respectively. Possibly other "formae" of *F. oxysporum* are responsible, along with *F. solani*, for the wilt and foot rot condition commonly observed in *Hibiscus* spp. and *Crotalaria* spp. *F. stilboides* is apparently responsible for a berry decay of *Coffea liberica* but it is possibly of minor significance unless it attacks the more economically important *Coffea arabica* L. and *C. robusta* L. Linden. *F. semitectum* and *F. moniliforme* are commonly encountered on maturing fruits, seeds, and other parts of the inflorescence of many plants and may be detrimental to some extent. Their entry into some plants is undoubtedly aided by commonly occurring injuries caused by insects. As *F. equiseti* was isolated only once, from soil, it is apparently too scarce to be of any importance in crop production.

The *Fusarium* flora of Trinidad presents some interesting differences from that of Canada, particularly with respect to the prevalence of some species and the apparent rarity or absence of others. *F. semitectum*, for example, was frequently encountered in various habitats whereas *F. equiseti* was found but once. In Canada, on the other hand, *F. equiseti* is commonly encountered in various habitats and *F. semitectum* is seldom found. As the geographic distribution of these two species is not known to be dependent on specific host plants, it is possible that each has become adapted to different climatic conditions. The apparent absence of certain species in Trinidad is also noteworthy because of their rather common occurrence in Canada and in many other parts of the world. These species are *F. poae* (Pk.) Wr., *F. avenaceum* (Fr.) Sacc., *F. acuminatum* Ell. & Ev., *F. culmorum* (W. G. Sm.) Sacc., and *F. graminearum* Schwabe. Evidently these species are not endemic in Trinidad. It is likely, however, that in the course of time some of them will be introduced by way of seed or by soil attached to the roots of plants.

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THE INFLUENCE OF ASSOCIATED MICROORGANISMS ON THE PATHOGENICITY OF HELMINTHOSPORIUM SATIVUM[1]

By W. P. Campbell[2]

Abstract

Six species of soil fungi were studied in association with *Helminthosporium sativum*. *Phoma humicola*, *Epicoccum purpurascens*, and *Trichoderma viride* strongly inhibited the pathogenic activities of *H. sativum*, while *Actinomucor repens*, *Sclerotinia trifoliorum*, and *Myrothecium verrucaria* were only slightly inhibitory. The pathogenicity of *H. sativum* was increasingly depressed by each of the six fungi as soil temperature was increased from 15° to 26° C. The pH shifts which these fungi induced in soil were not sufficient to be considered as a factor in depressing pathogenicity. In plate tests on nonenriched soil-extract medium, *S. trifoliorum* and *A. repens* did not appreciably affect *H. sativum*. The other four fungi all caused distortion and breakdown of the spores, while *M. verrucaria* and *E. purpurascens* were found as internal parasites in spores of *H. sativum*. *P. humicola*, *E. purpurascens*, and *T. viride* caused the breakdown of mycelium of the pathogen, and *P. humicola* and *E. purpurascens* were found as internal parasites of the mycelium. The fungi produced substances in the medium which adversely affect the germination and growth of germ tubes of spores of *H. sativum*. *A. repens* and *S. trifoliorum* were least effective, *T. viride* and *P. humicola* were intermediate in their action, and *E. purpurascens* and *M. verrucaria* were very severely limiting. Two antagonistic mechanisms, antibiosis and direct parasitism, were demonstrated, both of which were responsible for disorganization of the mycelium of the pathogen. Only those fungi that caused disruption of the mycelium were able to depress the pathogenicity of *H. sativum* appreciably.

Introduction

One of the most interesting developments in plant pathological research in the first half of this century has been the attempt to elucidate the bionomics of fungus associations, especially as they affect pathogenicity. Association effects are important, at least during the period when infection is initiated. This is particularly true with soil-borne pathogens where the diverse populations and close association present many opportunities for interaction.

From studies of such soil-borne pathogens as *Actinomyces scabies* (Thaxter) Güssow (18), *Erwinia carotovora* (Jones) Holland (8), *Ophiobolus graminis* (Sacc.) Sacc. (24, 28), *Fusarium culmorum* (W. G. Smith) Sacc. (14, 19), *Helminthosporium sativum* Pamm., King, & Bakke. (21, 23), *Rhizoctonia solani* Kühn (11, 26), and *Sclerotium rolfsii* Sacc. (20), we have convincing evidence that pathogenicity may be influenced by other microorganisms in the environment. The microbiological balance in the soil has been effectively displaced by the addition of amendments such as crop residue (10, 24), dried blood (16), soybean meal (3), dextrose (27), acetic acid (16, 27), and various soil fumigants (15, 25).

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Work to date has indicated that antagonism among microorganisms may be due to one or more of the following factors: competition for specific nutritive substrates; sequential colonization of constantly changing substrates as organic residues are broken down in the soil; rhizosphere effects induced by the physiological activity of roots; such factors as pH reaction, concentrations of oxygen and carbon dioxide, moisture, and temperature; parasitic activities of microfloral components; and the action of toxic metabolites or antibiotics. The importance of some of these factors is the basis for the work reported herein.

Methods and Results

A soil sample was taken from the root-rot nursery at this laboratory, and fungi were isolated from it by the dilution plate method. An acidified medium of the following formulation was used: 0.5 gm. yeast extract, 0.5 gm. peptone, 15 gm. dextrose, 15 gm. agar, and 1 liter of aqueous soil extract. The fungi isolated were transferred to tubes of the same medium.

Twelve fungi were selected at random for preliminary testing as to their effect on the pathogenicity of *H. sativum*. These fungi were grown in tubes of sterile soil to which 1% by weight of corn meal had been added. Cultures of an albino strain of *H. sativum* were also grown on this medium. Each of the test fungi was mixed with *H. sativum* and added to a flask of sterile soil at the same time that 10 surface-sterilized seeds of Thatcher wheat were planted. A similar series employed unsterilized soil. Four control groups were used as follows: *H. sativum* in sterilized soil, *H. sativum* in unsterilized soil, each test organism in sterilized soil, and each test organism in unsterilized soil. Each series was replicated four times and the entire test was repeated three times. All plants were grown for three weeks at 20° C. before they were examined to determine disease development. Each plant was assigned a value on a scale ranging from zero to 10, depending upon the severity of lesioning. Noninfected and killed plants received ratings of zero and 10, respectively, while various intermediate degrees of root and culm damage were indicated appropriately. Table I shows the percentage of disease caused in both the sterilized and the unsterilized soil. The results of the three tests were similar and have been averaged in the table.

From the fungi listed in Table I, the following six were chosen for further study: *Epicoccum purpurascens* Ehrenb., *Phoma humicola* Gilman & Abbott, *Trichoderma viride* Pers. ex Fr., *Actinomucor repens* Shostakovitsh, *Myrothecium verrucaria* (Alb. & Schw.) Ditmer ex Fr., and *Sclerotinia trifoliorum* Erikss. The first three of these were much more effective in reducing the pathogenicity of *H. sativum* than were the last three. Such a range made it possible to compare extreme reactions.

Influence of Temperature on Fungus Interaction

Previous studies have paid little attention to the influence which varying temperatures may exert on the interactions of *H. sativum* with other soil microorganisms. This factor was studied in the following experiment.

TABLE I

INFECTION OF THATCHER WHEAT SEEDLINGS BY *Helminthosporium sativum* ALONE AND IN COMBINATION WITH VARIOUS FUNGI

Fungus	Disease rating, %	
	Sterilized soil	Unsterilized soil
H. sativum alone	83	52
H. sativum plus		
Actinomucor repens Shostakovitsh	80	32
Cladosporium herbarum Link ex Fr.	87	34
Epicoccum purpurascens Ehrenb.	4	30
Gliomastix convolula (Herz.) Mason	43	48
Myrothecium verrucaria (Alb. & Schw.) Ditmer ex Fr.	59	40
Penicillium chrysogenum Thom	57	30
Penicillium puberulum Bainier	39	27
Phoma humicola Gilman & Abbott	28	31
Rhizopus nigricans Ehrenb.	49	45
Sclerotinia trifoliorum Erikss.	60	32
Stemphylium verruculosum Zimm.	54	33
Trichoderma viride Pers. ex Fr.	19	28

Four temperatures, 10, 15, 21, and 26° C., were maintained in "Wisconsin" tanks in a greenhouse. Six-inch pots of soil were autoclaved for three hours, then placed in the temperature tank containers and surrounded with clean sand. Inoculum of the test organisms and of *H. sativum* grown in soil to which 1% dextrose by weight had been added was mixed with the soil in the pots at the same time that 20 surface-sterilized seeds of Thatcher wheat were planted in each pot. After three weeks of growth the plants were taken up and rated for disease. This experiment was repeated three times.

From Fig. 1 it is apparent that with *H. sativum* alone or with any test fungus paired with *H. sativum* the incidence of infection increased with an increase in the temperature, and that *H. sativum* alone produced more severe infections at all temperatures tested than did any of the mixtures of the other fungi with *H. sativum*. At 10° C., growth of the wheat was very slow and at 26° it was etiolated. At 15 and 21° C. growth was good, and the disease picture was similar to that shown in the preliminary tests. Fig. 2 shows graphically the percentage reduction in infection produced by each of the test organisms.

pH Change in Relation to Fungus Interaction

Interactions characterized by zones of inhibition have long been recognized between fungus colonies growing on the same plate (9), sometimes between two colonies of the same species, and sometimes between those of different species. It has been assumed that such antagonism is due to substances excreted into the substrate by the organisms concerned. Since fungi are sensitive to pH changes in the substrates on which they grow, the possible role of this factor in the interactions between *H. sativum* and the test saprophytes was investigated.

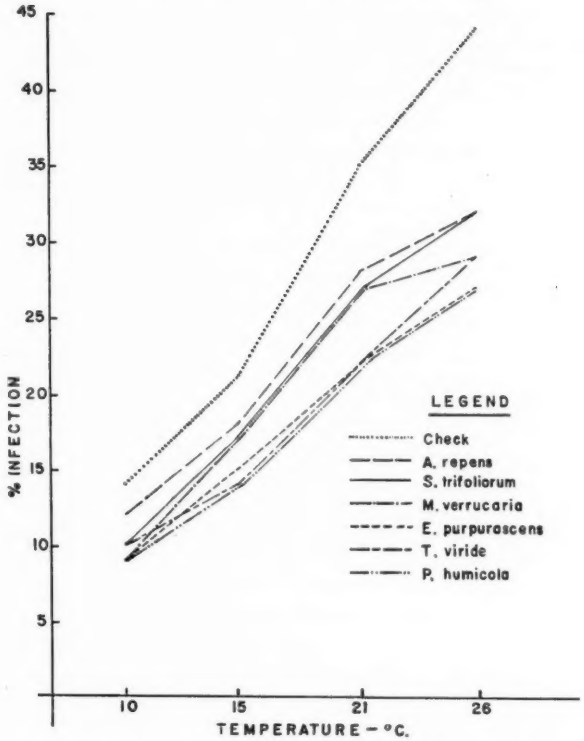


FIG. 1. Infection by *H. sativum* alone and in combination with other organisms at four different temperatures.

The test organisms were grown in flasks of steam-sterilized loam soil. In half of the flasks the soil was unamended and in the other half amended by the addition of 1% dextrose by weight. The flasks of sterile soil were inoculated with aqueous spore and mycelium suspensions and incubated at 20° C. for three weeks. The pH of the soil was then determined with a Beckman meter. Each treatment was replicated four times and the entire test repeated three times. There was abundant fungus growth, particularly in the soil with added sugar. None of the test organisms was able to alter appreciably the pH of the soil in which it grew, the greatest change being 0.3 of a pH unit.

Antagonism and Parasitism in Culture Plates

It was thought that a study of *H. sativum* growing on culture plates along with each of the test organisms might elucidate possible inhibition mechanisms and that if there were either a direct parasitic or a chemical effect on *H. sativum*, these would be demonstrable under such conditions.

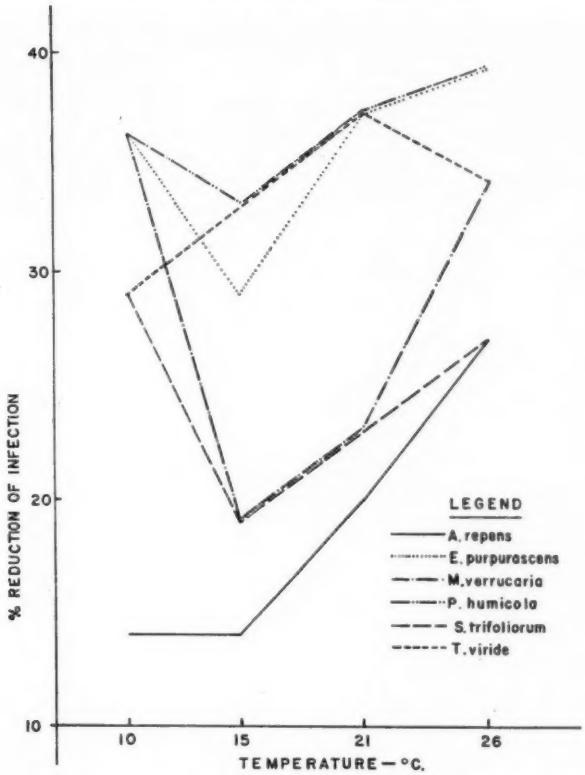


FIG. 2. Reduction in pathogenicity of *H. sativum* caused by other organisms at four different temperatures.

In most of these experiments a medium made up of 15 gm. of agar in 1 liter of aqueous soil filtrate was used. *H. sativum* and the test fungi were plated about two centimeters apart on the surface of the medium. All organisms sporulated freely, and, in fact, this was the only medium on which *S. trifoliorum* did fruit.

A. repens grew rapidly with no zone of inhibition between itself and *H. sativum*, which it overran without appreciable effect on either fungus. *H. sativum* grew slightly more slowly than in pure culture but fruited normally and eventually grew across the entire plate.

M. verrucaria grew freely through the colony of *H. sativum* and completely stopped the radial growth of the latter fungus. Microscopic examination revealed that *M. verrucaria* was actually parasitic upon *H. sativum*. Figs. 3, 4, 5, and 6 show progressive stages in the parasitic colonization of a spore of *H. sativum*. This fungus was also able to affect adversely the spores of the pathogen by action at a distance as depicted in Figs. 7 and 8. The cells of

a spore of *H. sativum* such as those shown in Fig. 8 could be separated by tapping the cover glass with a needle. Two separated cells are shown in Fig. 7.

E. purpurascens halted the radial growth of *H. sativum* colonies, was able to invade them, and caused a nearly complete breakdown of the mycelium and spores. Figs. 9 and 12 show direct parasitic attack on *H. sativum* spores, and Fig. 11 shows the remains of *H. sativum* mycelium. Attack on the spores of the pathogen seems to be associated with coiling of the attacking mycelium around its host, as is well demonstrated in Fig. 12.

P. humicola was able to inhibit the expansion of *H. sativum* colonies though its own rate of growth was greatly reduced by the association. Action against the spores of the pathogen was common and generally in advance of the mycelium, thus suggesting chemical diffusion. Figs. 10, 13, and 14 show the results of such action. Mycelial coiling or looping of *H. sativum*, as shown in Fig. 15, was also common. There was some evidence also of direct parasitic attack on the mycelium.

T. viride inhibited growth of the colonies of *H. sativum* but grew over them only after it had overrun the rest of the plate. When overgrown by *T. viride* the colonies of *H. sativum* were almost completely destroyed. No internal parasitism of the spores or mycelium of *H. sativum* was apparent, even when the mycelium of the pathogen was completely fragmented. Young spores failed to form septa and the cytoplasm was often extruded (Fig. 16) while older spores were disrupted and collapsed (Fig. 17). Spores that formed under the influence of action in advance of *T. viride* lacked proper spore walls and the cells became rounded (Fig. 18). The mycelium of *H. sativum* that was attacked was broken up and the cytoplasm was coagulated (Fig. 20).

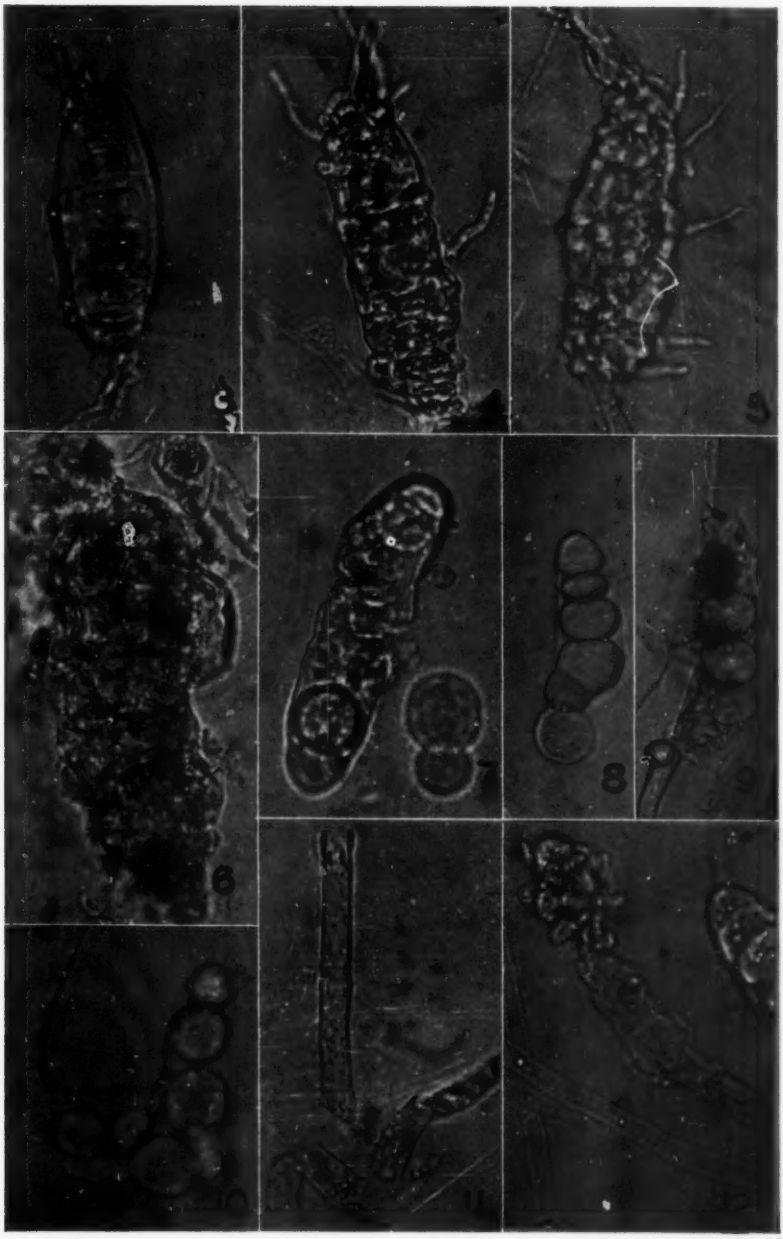
S. trifoliorum plated with *H. sativum* formed a permanent zone of mutual inhibition between itself and the other fungus. Aside from slight distortion of adjacent mycelium, it had no visible effect on the pathogen.

Effects of Diffusible Metabolic Products of Other Fungi on the Growth of Helminthosporium sativum

The production of antibiotic substances has been postulated as a mechanism by which one microorganism might affect the growth of another (9). The foregoing experiments suggest that such compounds were indeed active between some of the test organisms and the pathogen under consideration. The following tests were set up to further elucidate the mechanism.

FIGS. 3, 4, 5, 6. Progressive stages in the colonization of spores of *H. sativum* by *M. verrucaria*. FIG. 7. A mature spore of *H. sativum* breaking down under the action of diffusion products from *M. verrucaria*. FIG. 8. Distortion of a spore of *H. sativum* caused by diffusion products from *M. verrucaria*. FIG. 9. A spore of *H. sativum* parasitized by *E. purpurascens*. FIG. 10. Distortion of spores of *H. sativum* caused by diffusion products of *P. humicola*. FIG. 11. *H. sativum* mycelium fragmented by the action of *E. purpurascens*. FIG. 12. Mycelium of *E. purpurascens* coiled around a broken spore of *H. sativum*.

PLATE I



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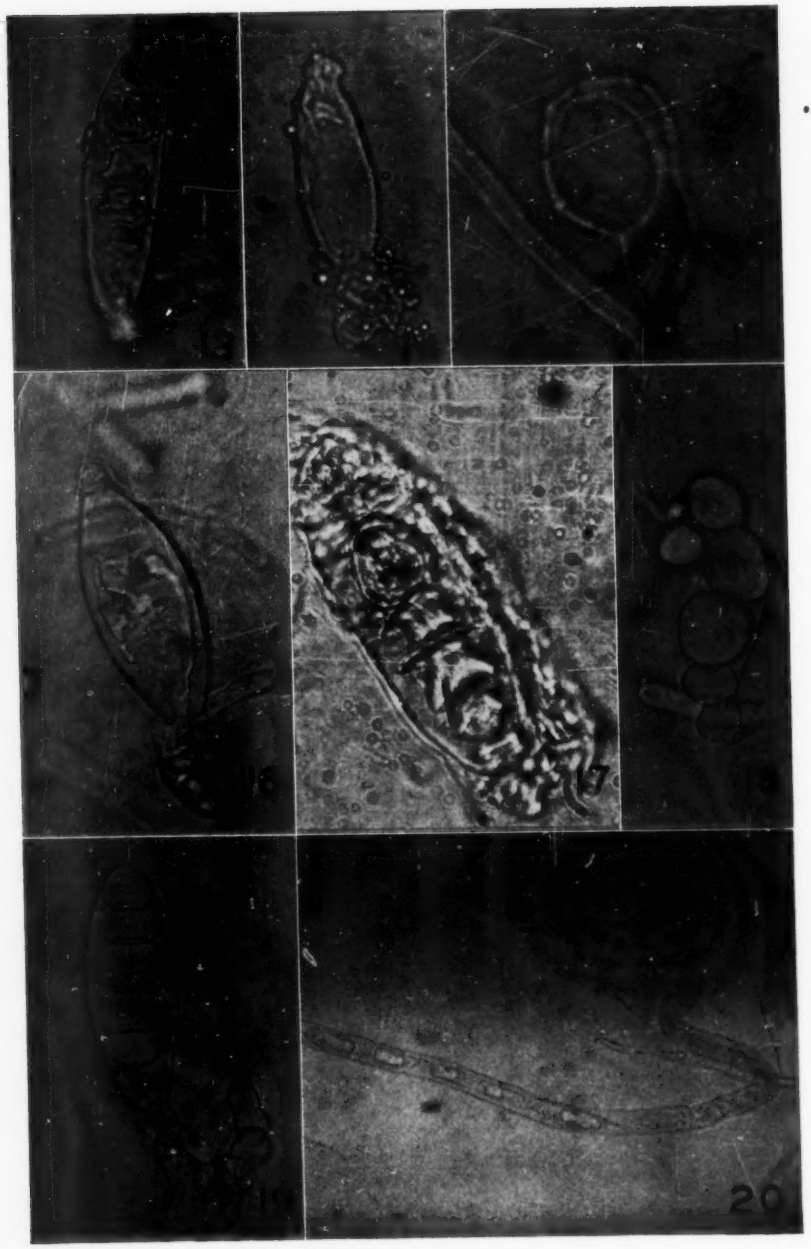

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The first tests were carried out on a soil filtrate agar medium enriched with 10 gm. of dextrose per liter of soil extract. The method used was that described by Jefferys (12). Van Tieghem cells 4 cm. in diameter and 1 cm. deep were placed in Petri dishes and poured full of agar medium. The upper surface was inoculated with the test organisms and incubated for six days at 20° C. The cells, complete with medium, were then turned over with a sterile spatula and the new upper surface was seeded with a water suspension of *H. sativum* spores. The plates were incubated at 20° C. for 24 hr. and the length of the germ tubes of *Helminthosporium* spores was measured. Twenty-five spores from each plate were selected at random for germ-tube measurement and 20 plates of each test organism were examined. Figures are not available to show the length of germ tubes when *H. sativum* spores were seeded on a control medium which had not borne another fungus previously because the growth in 24 hr. was so great that individual hyphae could not be traced. The results are summarized in Table II.

All test organisms reduced germ tube growth. Since the fungi had been grown on the side of the agar disk opposite that on which the *H. sativum* spores were seeded, the agent or agents which caused the decrease in growth rate must have diffused through the medium.

TABLE II

LENGTH OF GERM TUBES OF *H. sativum* SPORES INCUBATED FOR 24 HR. AT 20° C. ON AGAR CONTAINING DIFFUSION PRODUCTS OF VARIOUS TEST ORGANISMS

Test organism	Germ tube length (μ)
A. repens	831.8
S. trifoliorum	511.3
P. humicola	185.3
T. viride	62.2
E. purpurascens	18.5
M. verrucaria	5.2

After demonstration of antibiosis on an artificial medium it seemed desirable to determine whether it occurred in soil. Soil to which 1% dextrose by weight had been added was placed in Petri dishes and autoclaved for 30 min. The soil was inoculated with 10 ml. of aqueous suspensions of spores and mycelium of the test organisms. The plates were incubated at 20° C. for three weeks and then sterilized by autoclaving for 30 min., after which a 1% dextrose, soil-extract, agar medium was poured over the soil in each plate. The plates

FIGS. 13, 14. A mature and an immature spore of *H. sativum* showing signs of disorganization as a result of substances produced by *P. humicola*. FIG. 15. A loop formed on what appeared to be a young conidiophore of *H. sativum* associated with *P. humicola*. FIGS. 16, 17. An immature and a mature spore disorganized by diffusion products of *T. viride*. FIG. 18. Distortion of spores of *H. sativum* caused by diffusion products from *T. viride*. FIG. 19. Normal conidium and portion of a conidiophore of *H. sativum*. FIG. 20. Coagulation of the cytoplasm and fragmentation of the mycelium of *H. sativum* by *T. viride*.

were inoculated at the center with loops of a spore suspension of *H. sativum*. Controls consisted of plates of soil, similarly treated but not inoculated with test fungi. After the fungus had been incubated for one week at 20° C. the average diameter of each colony was determined. Only *M. verrucaria* had any appreciable effect; it reduced the diameter of the colonies by about 20% below that of the checks. In fact, four of the fungi showed a tendency to stimulate growth of the pathogen. There were, however, other observable effects on growth. In plates on which *T. viride* had been grown the colonies of *H. sativum* were more closely appressed to the medium and more compact than in any other treatment; the mycelium was excessively branched, and the spores were distorted. Some spores lacked outer spore walls and the swollen cells were easily separated. Where *P. humicola* had been used the same type of spore distortion was noted in *H. sativum*. *P. humicola* depressed sporulation.

Unfortunately the method used heat sterilization following the growth of the test organisms, which may have deactivated any thermolabile products of their metabolism.

Discussion

Three species of fungi, *T. viride*, *E. purpurascens*, and *P. humicola*, sharply reduced the pathogenicity of *H. sativum* and were able to destroy the spores and mycelium of the pathogen. The three species *A. repens*, *M. verrucaria*, and *S. trifoliorum* showed little ability to reduce the pathogenicity of *H. sativum* and had little effect on its vegetative mycelium, although *M. verrucaria* was very active against the spores of the pathogen. All test fungi produced diffusible toxic products that inhibited germ tube growth of *H. sativum* spores or caused distortion of its mycelium across the zone of inhibition on agar medium. However, there was no apparent relationship between the ability to produce diffusible toxins and the ability to reduce pathogenicity of the pathogen.

It is probable that the antibiotics produced by the six fungi were all different chemically and it is not surprising that different effects on *H. sativum* were observed. Several investigators, of whom Millard and Taylor (18) were among the first to publish, have reported on the fate of various antibiotics in soil. Gottlieb *et al.* (4, 5, 6, 7, 17, 22) report that the basic antibiotic, streptomycin, is readily adsorbed on soil colloids, while neutral and acidic antibiotics are relatively unaffected. Brian (1) states that there are indications that certain antibiotics are quite stable in neutral soil, and Gottlieb *et al.* (6, 7) report that chloromycetin and clavicin are stable in sterile soil but rapidly degrade in natural soil. Jefferys (13) reports that albidin, gladiolic acid, griseofulvin, and streptomycin are adsorbed in soil, that frequentin, mycophenolic acid, patulin, and penicillin are biologically deactivated in soil, while gliotoxin and viridin are deactivated at pH levels above 5, and penicillin at pH levels below neutrality.

M. verrucaria may, at first glance, appear out of place in the relatively ineffective group. It was noted, however, that although it was a vigorous parasite on the conidia of *H. sativum*, it was not parasitic on the mycelium. The inoculum used in pot tests was grown in soil where there was abundant mycelial development and thus *M. verrucaria* would be naturally less effective against the pathogen than those fungi that could destroy the mycelium. *T. viride* was not found to be an internal parasite of *H. sativum* in the plate tests, but it did cause breakdown of the spores and mycelium. It is known to produce an antibiotic, gliotoxin (2), which has been shown by Jefferys (12) to be relatively slowly broken down in soil. If gliotoxin can be produced in this environment then it possibly has some biological importance and may be the agent by which *T. viride* depresses the pathogenicity of *H. sativum*.

It seems, then, that only those fungi which caused the breakdown of *H. sativum* mycelium were able to curtail its parasitic activity severely. Two mechanisms have been demonstrated, a direct penetration of the cells of the pathogen by mycelium of *E. purpurascens* and *P. humicola*, and an antibiotic action by *T. viride*, both of which can disorganize the thallus of the pathogen.

Acknowledgment

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BEHAVIOR OF ALBINISM IN BROWN AND GRAY CULTURES OF THIELAVIOPSIS BASICOLA[1]

By R. H. Stover[2]

Abstract

Many single spore gray wild type cultures of *Thielaviopsis basicola* consistently produce an average of 3.5–4.2 albino sectors after 5–10 days growth at 73° ± 3° F. on potato dextrose agar and other media. Sectoring was greatly reduced at fluctuating room temperatures of 76°–87° F. and on certain media. Mass spore transfers from the albino sectors yield albino colonies with a small pigmented area around the center of transfer. Single spore cultures of the albino sectors inevitably revert to the gray parent. In contrast to the gray, brown type cultures rarely produce albino sectors or patches and these yield pure albino colonies that have remained stable for four years. All albino colonies are identical morphologically to the parents except in loss of pigment and a retarded thickening of the chlamydospore walls.

The expression of albinism in gray type cultures cannot be explained on the basis of a simple gene mutation. Also, an explanation based on a variable phenotypic change, originating in the cytoplasmic enzyme system controlling pigmentation and under constant genotypic control, is not entirely adequate. The expression of albinism in brown type cultures has the characteristics of a gene-type mutation.

Introduction

Sectoring is common among fungi grown on artificial substrates. Nearly always the formation of sectors has been attributed to a mutation or genic change. In the literature, however, there are reports of consistent sectoring in certain cultures, and in a few instances the sector has reverted to the parent when subcultured (2, 4, 6). Paxton (7) found a consistent "mutation" of *Helminthosporium* on a no-nitrogen medium, but there was no indication of reversion to the parent. Christensen (1) found that certain cultures of *Helminthosporium sativum* produced 8–10 sectors consistently at 27° C. Horne and Das Gupta (4) describe a cyclic type of sectoring and reversion in *Diaporthe perniciosa*. Sectors that reverted to the parent were termed "false sectors" (4). Such a progressive rate of "mutation" and reversion is not characteristic of genic mutation and indicates that some other mechanism of variation is operative. Leonian (6) studied variability in the genus *Fusarium* and refers to sectors as dissociations and not mutations. His work is weakened by the fact that single uninucleate spore cultures were seldom used. Nevertheless, his contention that genic mutations are not involved in a cyclogenic type of fungus variability but some factor inherent in the protoplasm was unique. An opportunity to study consistent sectoring to an albino and reversion to the pigmented parent was found in gray type cultures of *Thielaviopsis basicola*. The description of this sectoring and reversion, and possible mechanisms underlying such variability are presented.

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This work was begun at the Botany Department, University of Toronto and the Laboratory of Plant Pathology, Harrow, Ontario. The author acknowledges the generous assistance of the United Fruit Company in preparing the manuscript and completing certain experiments.
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Methods and Materials

Wild type cultures of *Thielaviopsis basicola* (Berk. & Br.) Ferraris were studied mostly on Difco dehydrated potato dextrose agar (P.D.A.) and other media previously described (11). Methods of handling cultures and the single spore (uninucleate) isolation technique were essentially the same as those in previous studies (9). Cultures were grown mostly on agar slants in 4 oz. bottles at room temperature (76°–87°F.) and at 73° ± 3°F.

Results

Many gray type single spore cultures of *T. basicola* consistently formed an average of 3.5–4.2 albino sectors when growing on potato dextrose agar and other media for 12 days at 73° ± 3° F. (Table I). Fluctuating and higher temperatures reduced the amount of sectoring, and some media were more favorable to sectoring than others (Table I). Sectors appeared as early as five days after transfer, but were most common after 7–10 days. The gray type albino sectors developed in culture tubes (Fig. 1, A), and in agar plates. Thus far, most gray type cultures tested from Virginia and other areas (9, 10) have yielded albino sectors.

When single spore cultures from the albino area in gray cultures were made, typical gray colonies inevitably developed, which after a period of 7–10 days produced one to six albino sectors. When multiple mass spore transfers were made from the albino sectors, the developing colonies were albino with the exception of a small pigmented area around the center of inoculation (Fig. 1, A). After a period of two to four weeks on the different media used, small pigmented clusters of chlamydospores were often visible in the albino portions of the colony (Fig. 1, D). In many cases, after a period

TABLE I

INFLUENCE OF TEMPERATURE AND MEDIA ON ALBINO SECTORING IN GRAY WILD TYPE CULTURES OF *Thielaviopsis basicola*

Agar media	pH	% of 50 colonies sectoring at: 76°–87°	73°	Average No. sectors per sectored colony at: 76°–87°	73°
Leucine-glucose	6.60	0	15	0	3.0
Arginine-glucose	7.00	35	95	1.1	4.2
$(NH_4)_2SO_4$-glucose	6.65	0	90	0	2.1
Asparagine-glucose	6.70	0	92	0	2.0
Leucine-sucrose	6.85	0	32	0	3.5
Arginine-sucrose	7.60	22	67	1	1.5
$(NH_4)_2SO_4$-sucrose	6.75	0	34	0	1.0
Asparagine-sucrose	6.85	0	84	0	2.0
Difco potato dextrose	5.30	45	95	2.5	3.5

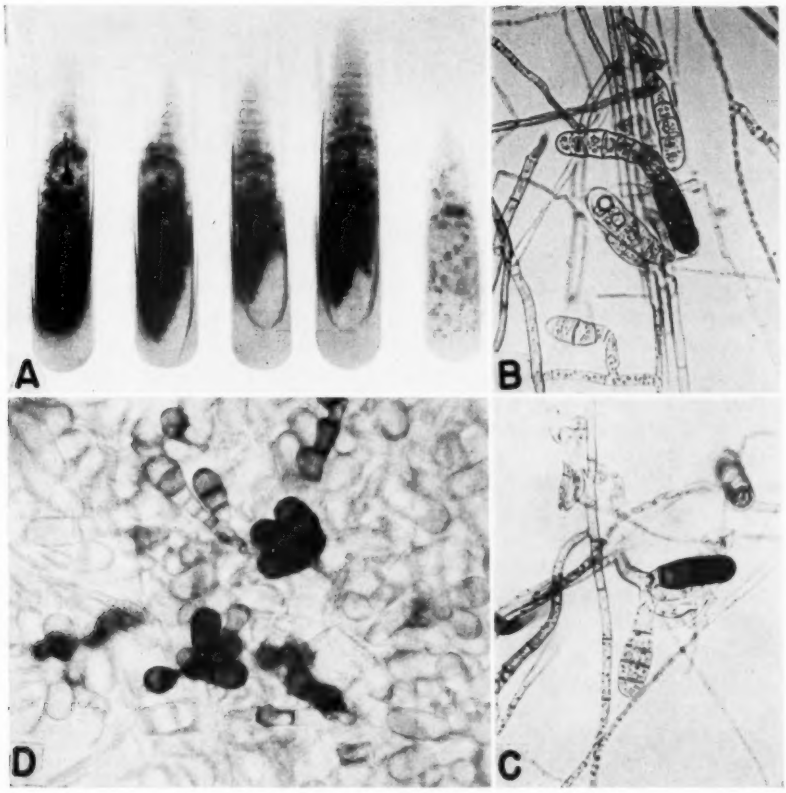

FIG. 1. A: A gray type culture (left) and three gray sectoring-to-albino single spore cultures (center). The albino culture (right) was derived by a multispore transfer from an albino sector in a gray colony. Note the dark portion near the center, which was the point of transfer and where pigmented chlamydospore and hyphae have developed.

B and C: Chlamydospores from the edge of an albino sector in a gray colony showing the presence of albino and pigmented chlamydospores in what appears to be "heterocaryotic" association. Note the thin walls of the albino, contrasted with the pigmented spores, some of which will later thicken.

D: The beginning of pigment formation in albino chlamydospores in a gray–albino culture as the culture ages. After two to three months on the media used all albino spores become pigmented.

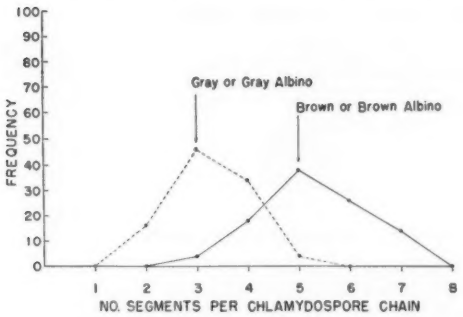

FIG. 2. Frequency distribution curve of number of segments per chlamydospore chain in gray or gray albino and brown or brown albino cultures.

of two to three months, the entire colony had become pigmented. Single or mass spore transfers from these "reverted" colonies yielded gray sectoring-to-albino colonies or albino colonies with pigmented centers respectively.

Since the expression of albinism in the gray type was influenced by the amount of inoculum used, observations were made on different methods of transfer. When loopfuls of conidia were streaked on plates of P.D.A., albino areas developed where the spore concentration was most dense and gray areas where the spore concentration was least. These gray areas later formed albino sectors. When hyphal tips from the edge of an albino colony (several pieces of hyphae often included) were transferred by a biscuit cutter technique, gray colonies developed which later formed albino sectors. When clumps of hyphae and conidia were removed in a biscuit cutter one-half inch back of the albino colony edge, albino colonies developed. Albino chlamydospores singly or in chains developed into gray colonies which produced albino sectors.

In contrast to gray type cultures, albino sectors or minute albino patches rarely formed in brown type cultures. Single spore cultures of the rarely occurring small albino patches or sectors from single spore brown type colonies yielded pure albino colonies which have remained stable for four years. In no case has there been any tendency of albino cultures from the brown type colony to "revert" to a pigmented form.

One of the morphological differences between gray and brown types is the number of segments per chlamydospore chain. An examination of albino chlamydospores from albino sectors derived from the brown and gray types shows (Fig. 2) that the albinos have the same average number of segments as the parents. No morphological difference between albino cultures and their wild type parents have been detected except the loss of pigment and a retarded thickening of the spore walls (Fig. 1, B and C). On the borders of the albino sectors, albino and pigmented chlamydospores were found in what appeared to be "heterocaryotic" association (Fig. 1, B and C).

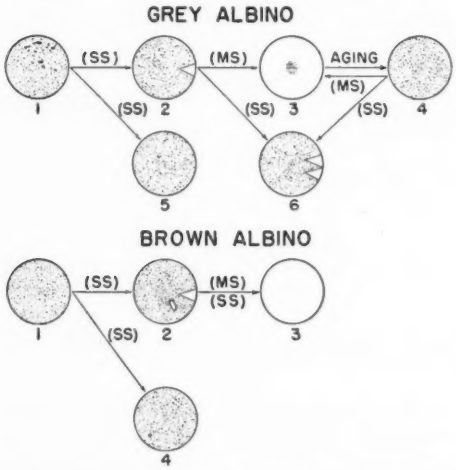

FIG. 3. Diagram summarizing the behavior of albino colonies from brown and gray wild type cultures; SS designates single spore cultures and MS multiple spore or mass inoculum cultures. Stippled areas represent pigmented areas and non-stippled areas are non-pigmented or albino. In the gray albino, No. 1 represents a young gray culture in which no albino sectors have yet developed; under certain conditions sectors do or do not develop consistently (Nos. 2 and 5). When single spore cultures are made from No. 2 either from the pigmented or albino sector a sectoring colony similar to the parent is obtained (No. 6). If a mass transfer of spores from the albino sector is made an albino culture with a small pigmented area in the center develops (No. 3). On aging this albino colony may become pigmented (No. 4). In the brown albino, No. 1 represents a brown culture which yields mostly non-sectoring cultures (No. 4) but a colony rarely develops with an albino patch or sector (No. 2). Single or mass spore transfers from the albino patch or sector in No. 2 yield pure stable albino cultures (No. 3).

Discussion

The behavior of albinism in *T. basicola* is summarized in Fig. 3. The consistent formation of albino sectors by gray *T. basicola* at certain temperatures and on some media, their inevitable reversion to the pigmented form or to an albino colony with mass transfer of inocula, and the gradual pigmentation of many albino colonies with age are contrary to all concepts of genic mutation. A mutation rate sufficiently high to account for albino sectoring is improbable unless some substance is produced which induces a non-genic "mutation" as in yeast (3). Even if a high rate of genic mutation was involved, the multiple reversions cannot be explained on this basis. An explanation of the phenomena observed might be based on a phenotypic change, such as could occur in the cytoplasmic enzymatic processes involved in pigmentation, with the genotype remaining intact. This temperature influenced change, or enzymatic "mutation", also might be influenced by unknown cultural conditions and/or an inherent gene controlled instability. The ability of the enzyme system to respond to these conditions is likely under gene control in the gray type, but such a plasticity is absent in the brown type. The instability of the enzyme system responsible for gray

type albinism is also indicated by the gradual conversion of some albino to pigmented colonies. Enzymatic change resulting in the formation of albinos appears to be quantitative as indicated by the results of single or mass spore transfers. The complete reversion to the original phenotype, under certain conditions of transfer, suggests some component of the colony or substrate in limiting concentrations and/or the diluting-out of the enzyme system responsible for albinism. A similar situation was indicated by Spiegelman (8) in the particulate transmission of enzyme forming capacity in yeast. Also, Jinks (5) has provided data on the reversible control of spore production in *Aspergillus nidulans* that could only be explained as resulting from non-genic or cytoplasmic changes.

The behavior of albinism in brown *T. basicola* is in strong contrast to the gray. It would appear that a genic mutation is involved in the former because of its relatively rare occurrence, lack of reversion, and stability over a period of four years. It is difficult to equate the extreme behavior of albinism in two wild type cultures of the same species. Apparently the gene controlled mechanisms involved in the formation of brown and gray type pigments are such that the gray type exhibits a phenotypic plasticity in response to environment absent in the former.

The role of a variable phenotype as a mechanism of adaptation in nature can only be speculated upon. Albinism in itself is of no parasitic advantage to the fungus (10). However, there may be other metabolic mechanisms that respond to an abnormal environment in nature in such a way as to favor survival, reverting to their original behavior when the environment is altered towards normality. Thus the phenotypic variation described may be only one of many possibilities resulting from a response of the organism to its environment, and in reality a form of adaptation.

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THE INFLUENCE OF LIGHT AND OTHER ENVIRONMENTAL FACTORS ON MYCELIAL GROWTH AND SCLEROTIAL PRODUCTION BY BOTRYTIS SQUAMOSA[1]

By O. T. Page

Abstract

A monoconidial isolate of *Botrytis squamosa* Walker was cultured on two semisynthetic media; modified White's medium permitted rapid and uniform mycelial growth; Czapek's medium supported abundant proliferation of sclerotia. When the fungus was cultured on White's medium and exposed to specific levels of incandescent irradiance, mycelial growth was arrested. The temperature inside Petri plates exposed to incandescent radiant energy was determined with the aid of thermocouples. Aeration and nutritional factors were found to influence the production of sclerotia. Concentric rings of sclerotia were produced on Czapek's agar when the fungus was exposed to periods of darkness alternated with periods of incandescent and/or fluorescent radiant energy.

Introduction

In 1925, *Botrytis squamosa* Walker was first distinguished from two related fungi, *B. allii* Munn and *B. byssoidea* Walker, and described as the cause of small sclerotial neck rot of the bulb onion, *Allium cepa* L. (9). Although others since then have studied the pathogenicity of *B. squamosa* on the leaves and bulbs of the common onion (3, 7), cultural investigations of the organism have been confined chiefly to the production of the perfect stage (1, 8).

In pathogenicity studies reported previously (4), discrete, circular to elliptical necrotic spots were induced on the inoculated leaves of onion plants when they were incubated in an environment with diurnal alternations of light. In comparison, when onion plants were incubated in the absence of light, infection was characterized by a rapid breakdown of leaves from their tips downward. It is apparent from these studies that parasitic action of *B. squamosa* was more extensive in continuous darkness than in alternate light and darkness. Although it was recognized that an onion plant requires visible radiant energy to photosynthesize, the influence of light on the fungus itself was unknown. The present paper is an attempt to resolve certain aspects of this latter effect. Observations were also made on the influence of light and other environmental factors on the pattern of sclerotial development *in vitro*.

Materials and Methods

A monoconidial isolate (S-190) of *Botrytis squamosa*, obtained from a leaf spot on an Early Yellow Globe onion grown on the Bradford Marsh, Ontario, was used throughout the experimental work. The cultural characteristics of this isolate on potato dextrose agar agreed in essential detail with *B. squamosa* as described by Walker (9).

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Contribution from the Botany Department, Ontario Agricultural College, Guelph, Ontario.

Two semisynthetic media were selected: one devised by White (10) permitted rapid and uniform mycelial growth; the other, a formulation of Czapek's agar medium (6), supported abundant proliferation of sclerotia. White's liquid medium was modified by the substitution of *d*-glucose, 10.0 gm., as the carbohydrate source, and by the addition of Bacto-agar, 17.0 gm.

Radiant energy was provided by either incandescent or fluorescent lamps. Since *B. squamosa* was grown in Pyrex Petri plates, all measurements of illuminance and irradiance were made with the Pyrex lids interposed between the energy source and the recording instruments. Illuminance was measured with a Weston Illumination Meter Model 756 which was cosine and color corrected. Irradiance was measured with a radiation thermopile connected to a Rubicon potentiometer calibrated in millivolts; 0.1 mv. was calculated to equal 14,000 ergs/sec./cm.^{2} Unless otherwise noted, all cultures were incubated at 23° ± 1°C.

Mycelial Growth

To investigate the influence of light on mycelial growth, seven Petri plates, 10 cm. in diameter, containing modified White's medium, were aligned with their diameters on a datum line. Beginning at a point in the center of the first plate in the series, each plate was inoculated at a point 8 cm. distant from the point of inoculation in an adjacent plate. A 25-watt incandescent lamp was positioned 15 cm. above the mycelial transfer in the first plate; illuminance was measured at each point of inoculation. Cultures exposed to continuous incandescent light and control cultures maintained in the absence of light were incubated for 120 hr. at 23° ± 1°C.

At the conclusion of the incubation period the extent of radial growth of a colony was found to be proportional to the distance of each colony from the incandescent lamp (Table I). The colony which was incubated at a

TABLE I

RADIAL GROWTH OF *Botrytis squamosa* EXPOSED TO INCANDESCENT LIGHT

Colony No.	Distance from light source, cm.	Illuminance, ft-c.	Mean radial growth, mm.
1	15	150	<1.0
2	17	85	<1.0
3	22	50	10.0
4	28	35	30.0
5	35	25	35.0
6	43	20	38.0
7	50	15	44.0
8	58	10	48.0
Control	—	Dark	48.0

distance of 58 cm. from the lamp (measurements taken from the center of the colony) had an average diameter of 48 mm., whereas the colony 15 cm. from the lamp had less than one-fiftieth as much growth at the end of the 120 hr. incubation period. A much reduced radial growth was apparent in the colony 17 cm. from the lamp as compared with the colony 28 cm. from the lamp. This response of *B. squamosa* to incandescent radiant energy was reproducible in successive trials.

In the foregoing experiment incandescent light with an illuminance somewhat in excess of 50 ft-c. arrested growth of the fungus. Consequently an experiment was devised to evaluate more precisely the conditions which prevented hyphal growth on exposure to incandescent radiant energy. Pyrex glass Petri plates, 15 cm. in diameter, containing modified White's medium, were inoculated along their diameters at points 1 cm. apart. The 13 mycelial transfers in each plate were exposed to either continuous darkness or to continuous incandescent radiation. The radiant flux incident on the mycelial transfers was measured beneath the interposed Pyrex glass cover with a radiation thermopile – potentiometer. Following inoculation, control cultures and cultures exposed to continuous incandescent light from a single unilateral source were incubated for 120 hr. in a temperature cabinet maintained at 23° ± 1°C.

Relatively uniform radial growth of the 13 mycelial transfers in a Petri plate resulted when they were incubated in continuous darkness at a constant temperature (Fig. 1). In contrast, the radial growth of each mycelial transfer in Petri-plate cultures exposed to continuous incandescent radiation, at a constant temperature, was influenced by the irradiance received by each transfer (Fig. 2). At an irradiance greater than approximately 81,000 ergs/sec./cm.^{2} mycelial growth was not evident at the conclusion of 120 hr. incubation; at less than 76,000 ergs/sec./cm.^{2} growth was proportional to radiant flux per unit area. Within the limits of the experiment, 76,000 to 81,000 ergs/sec./cm.^{2} represented the critical range of irradiance in excess of which radial growth was not apparent.

The total radiation reaching the surface of the substrate was calculated to be 1019 ergs/sec./cm.^{2} per foot-candle; the visible portion of this radiation was 94 ergs/sec./cm.^{2} Since approximately 90 per cent of the radiant energy incident on a Petri plate was non-visible, i.e. infrared, it was essential to determine accurately the temperature within a Petri plate positioned beneath an incandescent lamp. Temperature within a Petri plate, at the surface of the substrate, was measured by a series of copper–constantan thermocouples fastened 3.5 cm. apart along a diameter (15 cm.) of the bottom portion of a Petri plate. Agar was poured into the plate to a depth such that 2 mm. of each thermocouple tip protruded above the agar surface. The Petri plate was then positioned in the controlled-temperature cabinet with the linear series of thermocouples oriented toward the light source. Thermocouples from inside, and adjacent to the plate outside, were connected to a pyrometer – potentiometer calibrated in centigrade degrees.

The temperature at the surface of the substrate within a Petri plate was compared with the temperature of the air surrounding the plate. Fifty-two paired readings were made through a range of temperatures, within the plate, from 24° to 36°C. The maximum deviation of the temperature at the agar surface from the temperature of the air surrounding the plate was found to be +1.25 C°. From previous observations it had been determined that the maximum and minimum temperatures for mycelial growth were ±17 C.° from the optimum of 23°C., when the fungus was cultured in darkness on modified White's medium. A temperature difference of 17 C.° above or below the optimum was required then to arrest the growth of the fungus, i.e. to permit less than 1 mm. of radial growth during the 120 hr. incubation period. It seemed unlikely that inhibition of mycelial growth, as obtained in colonies receiving certain levels of incandescent irradiation, was due to an increase in temperature (+1.25 C.°) over the incubation temperature of 23°C. Further, since the temperature at a point within a plate receiving maximum irradiation was only 0.75 C.° above the temperature at a point receiving minimum irradiation, such a slight temperature difference could not account for the extreme variation in the growth of the mycelial transfers.

Sclerotial Pattern

Variations in the pattern of sclerotial development were observed frequently in replicate Petri-plate cultures of *B. squamosa* on agar media. The causes of such variations are multiplex. The following observations on the pattern of sclerotial development are the result of studies in which an attempt was made to modify an initial environment gradually.

To select a medium on which an abundance of discrete sclerotia could be produced, the fungus was cultured on various plant decoction and semi-synthetic agar media. Of the media investigated the most abundant proliferation of sclerotia occurred on Czapek's agar medium as devised by Thom and Church (6). Czapek's agar medium or modification thereof was used in all subsequent investigations on sclerotial formation.

The initial pH of Czapek's agar medium as measured potentiometrically was found to be 7.3. To determine whether this represented a suitable initial pH for sclerotial production, the fungus was grown on Czapek's agar medium adjusted to the following initial pH levels: 3.8, 5.0, 5.9, 6.9, 7.3, 8.0, 8.9, and 9.7. Replicate Petri-plate cultures of isolate S-190 of *B. squamosa* were incubated at each level in continuous darkness for 14 days, at a temperature of 23 ± 1°C. At the conclusion of the experiment abundant sclerotial development had occurred in cultures with an initial pH of 7.3 or less; a marked reduction in sclerotial production occurred in cultures with

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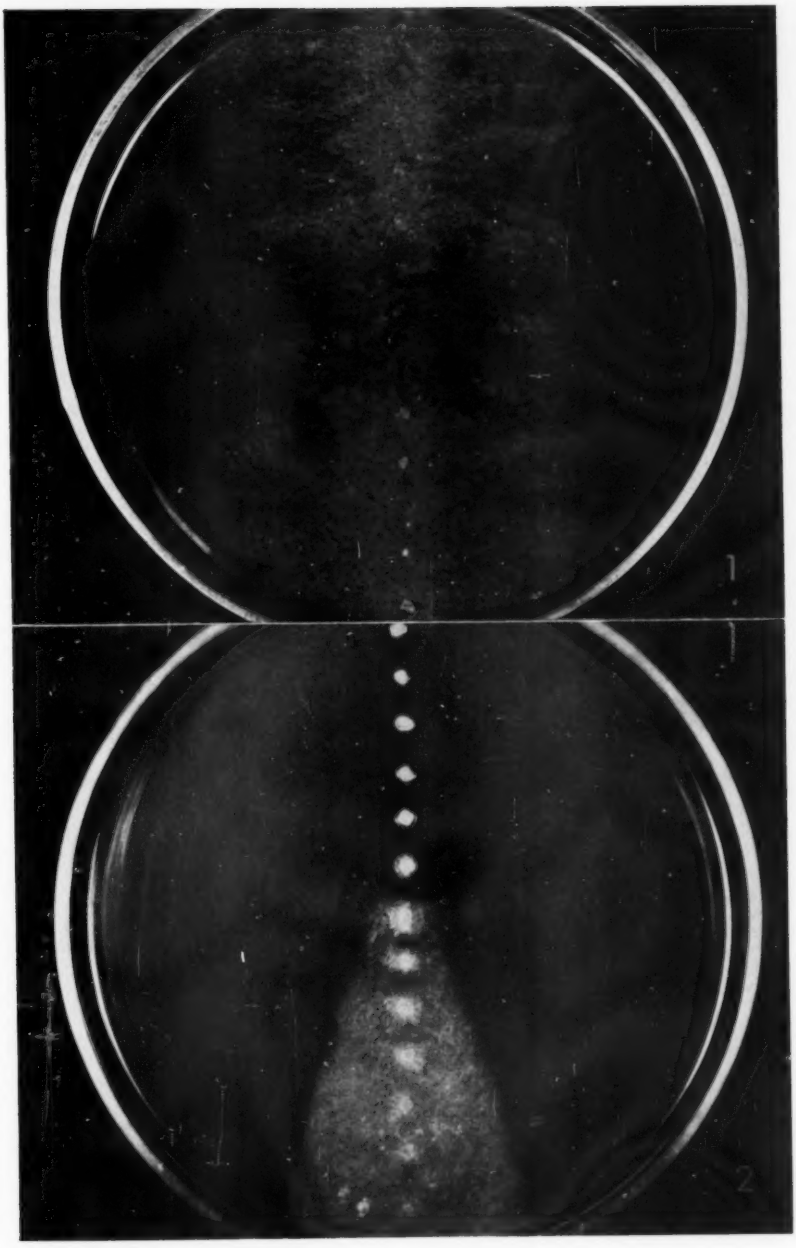
FIG. 1. Growth of 13 mycelial transfers of *B. squamosa* in continuous darkness.

FIG. 2. Growth of 13 mycelial transfers of *B. squamosa* exposed to continuous incandescent radiation. Irradiance at the center of the plate was approximately 76,000 ergs/sec./cm.^{2}.

PLATE I



an initial pH in excess of 8.0. In view of these results it was considered that abundant sclerotial production occurred on Czapek's agar medium over a relatively wide pH range; a pH range between 5.9 to 7.3 was considered to be satisfactory for sclerotial development in subsequent experiments.

Since information was lacking concerning the range of carbon and nitrogen sources which *B. squamosa* could utilize in sclerotial formation, the fungus was cultured on a variety of carbon and nitrogen sources. In experiments in which the carbon source was varied, the basal Czapek's medium contained sodium nitrate, 2.0 gm., dipotassium phosphate, 1.0 gm., magnesium sulphate, 0.5 gm., potassium chloride, 0.5 gm., ferrous sulphate, 0.01 gm., and Bacto-agar, 15 gm. per liter. The basal medium outlined above was modified by the addition of sucrose, 30.0 gm. per liter, and by the substitution of various nitrogen-containing compounds in place of sodium nitrate in experiments in which the nitrogen source was varied.

Twelve common sources of carbon were tested and the number of sclerotia produced on each source was determined. An amount of each carbon source to provide 12.6 gm. of carbon per liter of medium was used. The medium was adjusted to approximately pH 6.2, and all cultures were incubated in continuous darkness at a temperature of 23 ± 1°C. for 14 days. The results are presented in Table II.

It was found that abundant production of discrete sclerotia occurred on media containing the aldohexose monosaccharides *d*-glucose and *d*-mannose and on media containing disaccharides as the carbon source; *d*-glucose was common to the three disaccharides tested. Relatively few sclerotia were produced on the aldopentose monosaccharides *d*-xylose and *d*-arabinose and

TABLE II

PRODUCTION OF SCLEROTIA BY *Botrytis squamosa* ON MEDIA CONTAINING VARIOUS SOURCES OF CARBON

Carbon source	Amount of source,* gm./liter	Initial pH	Number of sclerotia†
d-Glucose	31.5	6.2	++++
d-Mannose	31.5	6.3	++++
Sucrose	30.0	6.3	++++
Lactose	31.5	6.3	+++
Maltose	31.5	6.3	+++
d-Xylose	33.6	6.1	+
Mannitol	31.8	6.2	+
Sorbitol	31.8	6.2	+
d-Arabinose	33.6	6.1	+
d-Galactose	34.6	6.3	0
d-Fructose	31.5	6.1	0
l-Rhamnose	33.6	6.1	0
Control	——	6.2	0

**Amount of source to provide 12.6 gm. of carbon/liter of medium.*
†Average of three replicate plates in two trials:
++++ = 300+; +++ = 200–299; ++ = 100–199; + = 1–99 *sclerotia.*

TABLE III

PRODUCTION OF SCLEROTIA BY *Botrytis squamosa* ON MEDIA CONTAINING VARIOUS SOURCES OF NITROGEN

Nitrogen source	Amount of source,* gm./liter	Initial pH	Number of sclerotia†
Sodium nitrate	2.00	6.1	++++
Potassium nitrate	2.38	6.1	+++
Urea	0.71	6.3	++
Asparagine	1.55	6.2	+
Ammonium tartrate	2.17	6.1	+
Alanine	2.10	6.1	+
Leucine	3.09	6.1	+
Glycine	1.77	6.2	0
Sodium nitrite	1.62	6.0	0
Ammonium sulphate	1.55	6.0	0
Control	——	6.2	0

**Amount of source to provide 0.33 gm. of nitrogen/liter of medium.*

†Average of three replicates in two trials:

++++ = 300+; +++ = 200–299; ++ = 100–199; + = 1–99 *sclerotia.*

on the polyhydric alcohols. It is of interest that *d*-glucose may be derived by oxidation of *d*-sorbitol, and *d*-mannose by oxidation of mannitol; both oxidation products were excellent carbon sources for sclerotial production. Although *d*-galactose and *d*-fructose form a part of the dissacharides lactose and sucrose, respectively, no sclerotia were produced on media containing either of these hexose monosaccharides. Rhamnose, a common desoxy sugar found in plants, when used as a carbohydrate source did not support sclerotial formation.

Eleven different sources of nitrogen were tested for the production of sclerotia. The results presented in Table III show that an abundant production of sclerotia occurred on nitrate sources while few or no sclerotia were produced on other nitrogen sources tested.

In the foregoing experiments, carbon and nitrogen were substituted in amounts equivalent to that of sucrose and sodium nitrate respectively, as contained in Czapek's medium. To determine the essentiality in sclerotial formation of the other constituents in Czapek's medium, constituents of the complete medium were deleted. It was found that sclerotial production was maintained when magnesium sulphate, potassium chloride, and ferrous sulphate were deleted, singly or together, from the medium. With a basal medium consisting of sodium nitrate, 2.0 gm., dipotassium phosphate, 1.0 gm., and Bacto-agar, 15.0 gm. per liter, an abundant production of sclerotia was maintained when carbon was supplied at the rate of 12.6 gm. per liter by such carbon sources as sucrose, maltose, *d*-glucose, and *d*-mannose. More than 150 sclerotia per Petri plate (10 cm. size) were produced on media consisting of sucrose, 30.0 gm., dipotassium phosphate, 1.0 gm., and Bacto-agar, 15.0 gm. per liter when nitrate nitrogen was supplied at the rate of

0.33 gm. per liter; ammonium nitrogen at the same rate did not support sclerotial production. In the above experiments all Petri-plate cultures were incubated at 23° ± 1°C. in continuous darkness.

Throughout the experiments in which sclerotial production of the fungus was investigated in relation to nutrition, considerable variation was encountered in the number of sclerotia produced in replicate Petri-plate cultures of the fungus on a particular medium. These variations in sclerotial production were not attributable to the amount of medium in a Petri plate, to the order in which Petri plates were poured from a single source of medium, or to inoculation technique. It seemed apparent that the variations were due to differences in the incubation environment within a particular Petri plate. The most evident variation among replicate Petri plates was the relative aeration afforded within a particular plate when Petri-plate cultures were incubated in continuous darkness at a temperature of 23° ± 1°C.

To investigate the influence of aeration on sclerotial production, Petri plates containing Czapek's agar were inoculated with isolate S-190 and sealed by introducing liquid vaseline between the side walls of the upper and lower portions of each plate while the plates were in an inverted position. Other plates were aerated by placing a 22-gauge wire clip over the edge of the bottom portion of a plate to separate the upper and lower halves of a plate along one side. Five replicate plates, sealed and aerated, were incubated at each of five temperatures in continuous darkness. The results are presented in Table IV.

TABLE IV

EFFECT OF AERATION ON THE PRODUCTION OF SCLEROTIA BY *Botrytis squamosa*

Temperature, C.° ± 1°C.	Number of sclerotia*	
	Sealed	Aerated
12	0	0
17	3	146
22	0	243
27	0	83
32	0	0

**Average count made in five replicate Petri plates (10 cm.) after 14 days' incubation of the fungus in continuous darkness on Czapek's agar medium.*

From the results presented in Table IV it is apparent that aeration is an important factor in the production of sclerotia by *B. squamosa*. Other observations also suggest that adequate aeration is essential: sclerotia of *B. squamosa* are produced typically on the surface of a solid substrate; sclerotia develop as part of a plectenchyma on the surface of potato dextrose and onion dextrose decoctions, and on Czapek's liquid medium; sclerotia commonly develop on the surface scales of infected white onion bulbs even though hyphal penetration may extend into the center of the bulbs.

Factors such as nutrition, aeration, and temperature have been shown to influence the number of sclerotia produced by isolate S-190 *in vitro*. In addition the number of sclerotia was influenced by the genotype of the isolate. A single-spore isolate M-208 failed to produce sclerotia on a variety of media. Isolates S-208 and S-215 produced sclerotia in greater number but of smaller size than isolate S-190; the size of the sclerotia produced by isolate S-190 was average for the isolates examined in culture. Although Viennot-Bourgin (8) reported the development of apothecia on Czapek's agar, no ascocarps were observed on Czapek's or other media used in the present investigation.

Mycelial zonation (Fig. 3) was frequently observed when the organism was incubated in alternate light and darkness. The concentric annuli of profuse and sparse mycelial growth were most apparent on media, such as modified White's medium, which supported abundant vegetative growth of the fungus (in darkness). On media which supported abundant sclerotial formation, more or less distinct rings of sclerotia developed in response to a fluctuating environment.

To investigate the influence of alternate periods of light and darkness on the pattern of sclerotial formation, isolate S-190 was grown in Pyrex Petri plates on Czapek's agar medium; the initial pH of the medium was 7.3. The plates were aerated by the insertion of a wire clip between the upper and lower halves of a plate. Cultures were exposed to alternate periods of light and darkness in a light panel maintained throughout each 14-day incubation period at 23° ± 1°C. The light source in the panel consisted of 28 General Electric 96 inch T8 daylight fluorescent lamps which were operated in conjunction with 10 G.E. 60 watt incandescent lamps.

The duration of the alternate periods of light and darkness was set arbitrarily at 12 hr. Replication of specific conditions of irradiation in a series of Petri plates, while possible, is extremely difficult to achieve. As a consequence, the irradiation beneath the lid of each Petri plate in a particular trial was recorded. In replicate trials, Petri-plate cultures were oriented spatially to obtain similar irradiance.

The results of a typical trial are shown in Fig. 4. From (*a*) to (*i*) respectively, the centers of these cultures received the following levels of incandescent and fluorescent irradiance: 0, 140, 350, 370, 378, 434, 532, 560, and 658, × 1000 ergs/sec./cm.^{2}. It is evident that the most discrete rings of sclerotia were produced at an irradiance of 370,000 to 434,000 ergs/sec./cm.^{2}; a tendency toward ring formation is evident in plate (*c*) at an irradiance of 350,000 ergs/sec./cm.^{2}. Plates exposed to incandescent-fluorescent radiant energy with an irradiance in excess of approximately 500,000 ergs/sec./cm.^{2} produced few sclerotia; mycelial growth was retarded and was thick-appressed with little evidence of zonation.

FIG. 3. Mycelial zonation of *B. squamosa*.

FIG. 4. Sclerotial zonation of *B. squamosa*. Irradiance from (*a*) to (*i*), respectively, was 0, 140, 350, 370, 378, 434, 532, 560, and 658, × 1000 ergs/sec./cm.^{2}.

PLATE II

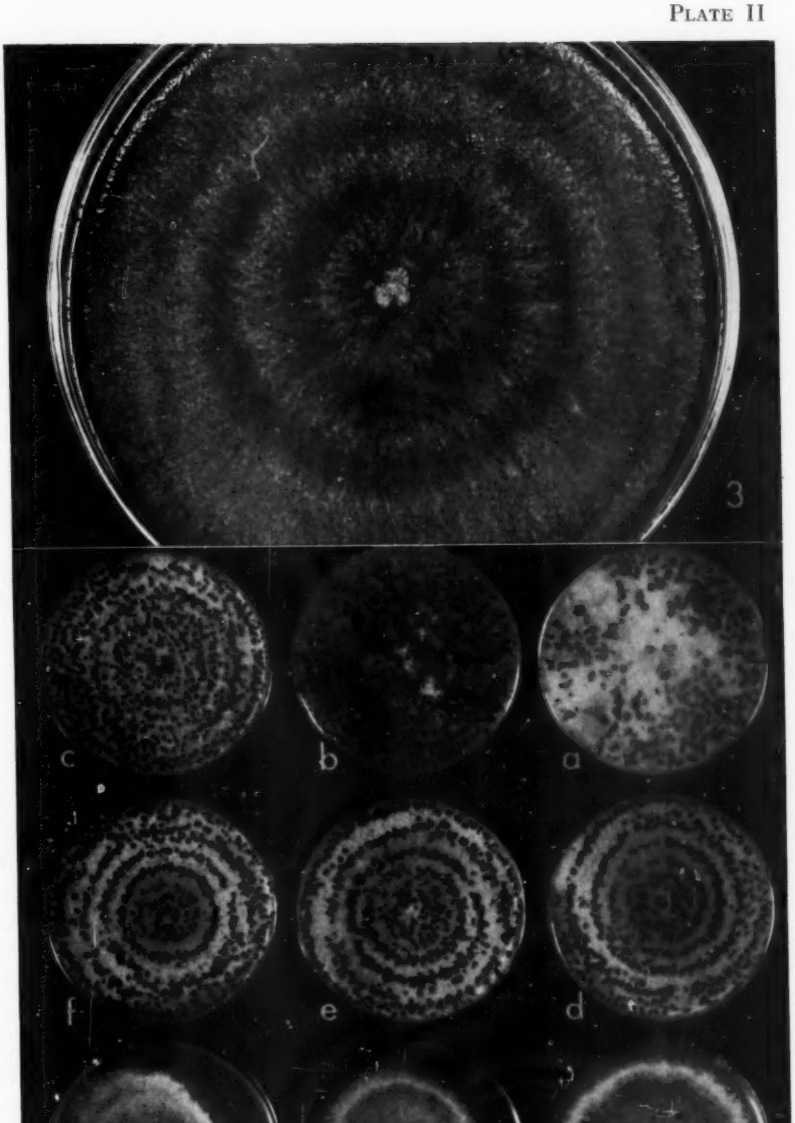


PLATE III

With radiant energy provided by incandescent and fluorescent lamps, much of the total irradiance was contributed by the incandescent sources; illuminance was largely attributable to the fluorescent lamps. When the incandescent sources of radiant energy were eliminated, illuminance in excess of 1000 ft-c. was obtained, but at a relatively low irradiance level. The results of exposure of cultures, similar to those used in the preceding experiment, to alternate 12-hr. periods of fluorescent irradiation and darkness are illustrated in Fig. 5. From (*a*) to (*d*) respectively the centers of the cultures received 0, 175, 210, and 336, × 1000 ergs/sec./cm.^{2}. It was possible to produce rather discrete rings of sclerotia in cultures intercepting relatively low levels of fluorescent radiant energy. Such results suggest that it may be largely the irradiance contributed by visible light that influences the pattern of sclerotial formation.

The exposure of *B. squamosa* to cyclic periods of visible radiant energy and darkness resulted in the subsequent development of rings of sclerotia. To determine if cyclic changes in temperature might produce a similar sclerotial pattern, Petri-plate cultures were maintained in continuous darkness and exposed to a temperature of 23°C. alternated with a temperature of 38°C. Nine different treatments with respect to exposure times were investigated. The most discrete concentric rings of sclerotia were produced by replicate cultures exposed to a temperature of 10°C. for 21 hr. alternated with a three hour exposure at 23°C. (Fig. 6).

Discussion

In a review of the effects of radiation on fungi (2) it was noted that the result of radiation experiments cannot be easily duplicated. Few general conclusions may be drawn because environmental factors have been so largely neglected. The converse may be also true. Environmental factors are often carefully controlled but the investigator fails to mention whether the fungus has been exposed during incubation to radiant energy in the near ultraviolet, visible, and near infrared regions of the spectrum. In the present investigation the sources contributing visible radiant energy, the measured irradiance, and incubation temperatures are in general duplicable. Illuminance readings were included to emphasize the apparent effect on the mycelial growth of *B. squamosa* of slight changes in luminous energy. Since approximately 90 per cent of incandescent radiant energy is non-visible, the eye or a selective detector such as an illumination meter gives little indication of the physiological response of the fungus to the total radiation from such a source.

In the past, considerable attention has been given to the response of fungi to the degree of heat which prevailed during incubation. The direct result

FIG. 5. Sclerotial zonation of *B. squamosa*. Irradiance from (*a*) to (*d*), respectively, was 0, 175, 210, and 336, × 1000 ergs/sec./cm.^{2}.

FIG. 6. Sclerotial zonation of *B. squamosa*. The culture was exposed to a temperature of 10°C. for 21 hr. alternated with a three hour exposure at 23°C.

of an increase in temperature is an acceleration of the velocity of enzymatic reactions until an optimum is reached; beyond an optimum temperature, some temperature eventually is reached where enzyme systems are permanently inactivated. Indirectly, the rate of growth of the fungus culture is influenced. The inhibition of mycelial growth of *B. squamosa* resulting from exposure of the fungus to incandescent radiant energy, at a certain irradiance level, is apparently a response to visible and/or near infrared radiant energy. The transmittance of Pyrex glass Petri-plate covers precluded irradiation of the fungus mycelium by wave lengths of ultraviolet radiant energy normally considered to be lethal.

Exposure of cultures of *B. squamosa* to incandescent or fluorescent sources of radiant energy alternated with exposure to a zero level of luminous energy resulted in mycelial and/or sclerotial zonation; the type of vegetative zonation was influenced by the medium on which the fungus was cultured. Exposure to artificial luminous energy decreased the velocity of certain enzymatic reactions; resumption of more or less normal activity of these reactions occurred during the dark period. Such abrupt changes in the environment with respect to radiant energy left an "imprint" on the fungus which became macroscopically apparent five to seven days later when rings of sclerotia developed. Apparently the level of irradiance which prevailed during the light-exposure period influenced the discreteness of the sclerotial rings which subsequently developed. Although the experiments were conducted under conditions in which temperature was rigidly controlled, it is difficult to eliminate the influence of light absorbed by the fungus and by the medium and transformed to heat.

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THE INHIBITORY ACTIVITY OF EXTRACT OF CAPSICUM FRUTESCENS ON PLANT VIRUS INFECTIONS[1]

By Colin D. McKeen[2]

Abstract

The expressed juice from *Capsicum frutescens* L. contains a substance that markedly decreases the formation of local and primary lesions by certain viruses on cowpea and *Chenopodium hybridum* L. and to a lesser extent on *Nicotiana tabacum* vars. Harrow Velvet and Samsun, and *Physalis peruviana* L. The extent to which pepper juice decreases the numbers of lesions is independent of the virus concerned and depends only on the species to which inoculations are made. Pepper juice inhibits the production of local lesions when it is applied with the virus inoculum, or to the upper and lower leaf surfaces before inoculation; but, if applied to leaf surfaces a few minutes after inoculation, pepper juice does not appreciably decrease production of lesions. Thus, it would seem that the effect of the inhibitive agent is to prevent infection by, rather than increase of, the virus. The application of the pepper extract at some distance from the point of virus inoculation also inhibits lesion formation on cowpea and *Chenopodium*. As judged by the reduction of lesions on cowpea, pepper extract displays about the same degree of inhibition as that of spinach, this being much greater than that of cucumber. The inhibitive component of pepper juice is thermolabile, it resists aging and drying *in vitro*, and does not pass through a cellophane membrane. Such characteristics indicate its protein nature. The protein component of pepper juice precipitated either by 95% ethanol or by ammonium sulphate is inhibitively active, but is less so than the raw juice.

Introduction

During the last twenty years a wide range of different substances has been shown by a number of investigators to interfere with the ability of viruses to infect susceptible plants. A comprehensive review of the investigations in this field has recently been made by Bawden (1). No chemical specificity of inhibitive substances is apparent, and some inhibitors are components of plant systems. Among such substances are sap of pokeweed (3, 4, 9), spinach (10), cucumber (12), *Dianthus barbatus* L. (15), and chenopodiaceous plants (8, 10). Crowley (2) has recently shown that substances inhibiting infection by cucumber mosaic and tobacco mosaic viruses occur in the seeds of these hosts.

A short time after a study was begun on the identification of aphid-borne viruses infecting sweet pepper crops in southwestern Ontario it became obvious that *Capsicum frutescens* L. contained a substance that inhibited the expression of virus symptoms in certain hosts. The failure to appreciate the significance of such an agent early in these studies resulted in some confusion in the identification of the viruses involved.

As was described previously by the writer (11), when juice expressed from the leaves of pepper plants known to be infected with cucumber mosaic virus (CMV) was rubbed on the primary leaves of cowpea, occasionally one or two discrete local lesions developed, and frequently no lesions appeared. In

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contrast, inoculum of the same virus from infected Harrow Velvet tobacco plants produced large numbers of lesions on every inoculated cowpea leaf. Three different isolates of CMV yielded similar results. It was shown further that when CMV infective juice from tobacco was adulterated to the amount of 5% with crude pepper sap the infectivity, as judged by local lesions on cowpea, was reduced by approximately 95%. Similar assays made on *Chenopodium hybridum* showed slightly more than a 90% reduction in local lesions. These results demonstrated conclusively the presence of an inhibitor or inhibitors in pepper extract. Furthermore, pepper extract was found to be capable of reducing the infectivity of CMV regardless of whether the virus was multiplied in *Nicotiana tabacum*, *Nicotiana glutinosa*, or in cucumber. Notwithstanding the presence of the inhibitor in pepper no difficulty was encountered in transmitting the virus from infected to healthy pepper. This finding is in general agreement with that of Gendron and Kassanis (7), who worked with different virus and host combinations.

This paper relates the inhibitive effect of pepper extract with other virus and host combinations, and also deals with the nature of the inhibitor and the mechanism of inhibition.

Experimental Results

Demonstration of Inhibition

Materials and Methods

Unpurified virus preparations were obtained by macerating the infected source-plant material in a mortar with a few drops of water. The infectious juice was strained through cheesecloth to remove tissue fragments. These extracts were diluted with a 1% phosphate buffer at pH 7 to obtain a virus solution that would produce sufficient countable lesions to permit a satisfactory analysis of infectivity. Plant extracts to be tested for inhibitive capacity were strained through cheesecloth and then were either added to the virus solutions or were applied to the leaf surfaces before or after inoculation. To inoculate the plant, leaves were first dusted lightly with carborundum then rubbed with the forefinger wetted with the test inoculum. The entire upper surface of each leaf lamina was stroked twice from base to tip. The leaves were rinsed with water immediately after inoculation. Except where specified otherwise the pepper extracts were obtained from the green leaves of the California Wonder variety.

Infectivity tests of the viruses were made, for the most part, on hosts that give countable necrotic primary or local lesions. The viruses tested and their assay hosts were as follows:

Cucumber mosaic virus (CMV); Price's strain 6; assayed by local lesion response on cowpea var. Blackeye and *Chenopodium hybridum* L.

Tobacco etch virus (TEV); a severe strain; assayed on the inoculated leaves of burley tobacco var. Harrow Velvet, and on cape gooseberry, *Physalis peruviana* L.

Tobacco mosaic virus (TMV); a green mottling strain; assayed on *Datura stramonium* L. and *Nicotiana glutinosa* L.

Tobacco ringspot virus (TRV); assayed on cowpea and Harrow Velvet tobacco. Another ringspot virus isolated from cucumber (CRV); assayed on *N. glutinosa*, *N. tabacum* var. Samsun, *D. stramonium*, cowpea, and *C. hybridum*.

Inhibitive Effect of Pepper Extract on Four Viruses

Preliminary infectivity tests with TMV and TEV showed that these two viruses differed in their apparent concentrations in infected pepper tissue. Expressed juice from pepper infected with TMV always produced numerous local lesions on *N. glutinosa*, and *D. stramonium*. Simultaneous inoculations of other susceptible hosts yielded 100% infection. In contrast, when sap from the leaves and fruits of pepper plants naturally infected with TEV was used to inoculate Harrow Velvet tobacco and *P. peruviana*, frequently 10% of the former and 50% of the latter failed to become infected. The suggestion that the inhibitive component of pepper extract operated against some but not all viruses warranted investigation. Consequently, tests were conducted to determine the effect of pepper extract on the infectivity of TMV, TEV, TRV, and CRV. Of necessity, this involved the use of several local and primary lesion hosts.

Physalis peruviana L. proved to be an unsatisfactory local lesion host for assay tests with TEV, because systemic invasion by the virus usually caused leaf abscission before primary lesions appeared. This host response occurred even when the experiments were conducted under the reduced light intensity of late autumn months. Nevertheless, the effect of adding pepper extract to infective tobacco juice was obvious, for it retarded the appearance of systemic symptoms by 24 or more hours. Furthermore, the amount of leaf abscission was not as extensive on these plants as on those inoculated with the unadulterated juice.

When young succulent leaves of Harrow Velvet tobacco were inoculated with TEV, primary lesions of the etch-ring type were sufficiently distinct to be readily counted. Consequently, this host was used for assay studies with this virus. Because CRV causes necrotic primary lesions on the leaves of a number of plants, some of which serve as local lesion hosts for other viruses, the results of inoculations with this virus were especially interesting.

The tested viruses were multiplied in Samsun tobacco plants and the expressed infective juice was adulterated with pepper sap at the rate of 1 volume of the latter to 30 volumes of the former. The expressed sap from pepper was frothy, and since it assumed a jelly-like consistency when allowed to remain in an open vessel for two or three minutes, the required aliquots were taken before this condition obtained.

An examination of the results presented in Table I shows that the adulteration of infective tobacco juice with pepper sap reduces the infectivity of viruses on some hosts but not on others. No inhibition occurred on either *Datura* or *N. glutinosa*. Pepper sap caused the greatest inhibition on cowpea

TABLE I

THE EFFECT OF EXPRESSED JUICE FROM *Capsicum frutescens* ON THE INFECTIVITY OF DIFFERENT VIRUSES

Virus	Assay host	Average no. of lesions per inoculated leaf		
		Unadulterated juice	Adulterated juice	Juice from infected pepper
TMV	*Datura*	414	398	
	N. glut.	358	337	324
TEV Expt. 1	H. V. tobacco	212	30.5	4
" 2	" "	290	41.1	4.2
TRV	Cowpea	135	0.1	
	H. V. tobacco	379	24	
CRV Expt. 1	*Datura*	943	838	
	N. glut.	12.8	14.5	
	Samsun tobacco	93.7	8.7	
	C. hybridum	238	50.1	
Expt. 2	*Datura*	650	631	
	N. glut.	39	36	
	Samson tobacco	35	4	
	C. hybridum	268	10	
	Cowpea	10	0	

and somewhat less on *C. hybridum*, H. V. tobacco, and Samsun tobacco. The adulteration of the TEV juice with pepper extract reduced the number of primary lesions on H. V. tobacco by about 85%. Inoculum obtained from infected pepper plants produced still fewer lesions, amounting to less than 2% of the number caused by infective tobacco juice.

Since pepper juice did not alter the response of *Datura* to infection by CRV the possible counteraction of pepper juice by *Datura* juice was suggested. This effect was studied by incubating the two juices in a vessel for 10 min. and then adulterating the CMV inoculum with a few drops of the mixture before inoculating cowpea. These tests showed that incubating pepper juice with *Datura* juice for a short time did not counteract the inhibitive power of the former.

The Comparative Inhibitory Capacities of Pepper, Spinach, and Cucumber Extracts Against CMV

Sill and Walker (12) showed that extracts of healthy cucumber plants contain a substance highly inhibitive to CMV 1. Kuntz and Walker (10) showed that crude spinach extract reduces the infectivity of TMV and cabbage mosaic virus. It seemed worthwhile to compare the inhibitive capacities of cucumber and spinach extract with that of pepper.

Extracts were made from green leaves of healthy spinach, cucumber, and pepper by macerating the leaves in mortars with sufficient water to obtain a few drops of the respective extract. Enough of the test extracts was added

TABLE II

INHIBITIVE CAPACITY OF EXTRACTS FROM SPINACH AND PEPPER TO CMV AS SHOWN BY ASSAYS ON COWPEA AND *C. hybridum*

	Inoculum	Average number of lesions per leaf*	
		Cowpea	*C. hybridum*
Expt. 1.	Infective juice (CMV)	98.4	177.5
	" " + pepper extract	1.8	18.0
	" " + spinach extract	2.0	163.0
Expt. 2.	Infective juice (CMV)	62.0	80.0
	" " + pepper extract	0.1	0.1
	" " + spinach extract	5.5	76.0

**Each type of inoculum was rubbed on 24 cowpea leaves and on six leaves of each of three C. hybridum plants.*

to the CMV juice from tobacco to obtain a 3% adulteration. The unclarified extract from minced pepper leaves was observed to be more viscous than that from cucumber and slightly more viscous than that from spinach. Preliminary bio-assays on cowpea revealed the inhibitory power of cucumber extract to be weak as compared with that of pepper and spinach. The results of further tests comparing the inhibitive capacities of extracts from spinach and pepper in which both *Chenopodium* and cowpea were used as assay hosts are shown in Table II.

The results presented in Table II show that the addition of pepper extract to CMV juice reduced lesion production on both cowpea and *Chenopodium* to a low level. In contrast, the spinach extract effected a reduction only on cowpea. It is perhaps unnecessary to mention that *Chenopodium* and spinach belong to the same plant family, and that extracts from members of the same plant family are not usually inhibitive.

Nature of Pepper Inhibitor

Distribution of the Inhibitor in the Pepper Plant

Tests were conducted to determine in what organs of the pepper plant the inhibitor resides, and also the concentration in each. For these purposes 1 volume of extract expressed from different organs of the pepper plant was added to 30 volumes of CMV-infective juice from tobacco.

The results presented in Table III show that the inhibitive substance is present in all the organs of the pepper plant. The extracts from the leaves and corolla were most inhibitive, whereas that from the roots was least inhibitive. The inhibitive capacity of the fruit and stem extracts was intermediate between that of leaves and roots. The results of a similar experiment in which *C. hybridum* was used as the assay host were in close agreement with those obtained on cowpea.

TABLE III

POTENCY OF THE INHIBITIVE EXTRACTS FROM DIFFERENT ORGANS OF PEPPER PLANT

Inoculum	Source of additive	Av. no. of lesions per inoculated cowpea leaf*			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
Infective juice†	Green leaf	1.1	1.3	8.7	9.7
" "	Senescent yellow leaf	—	1.0	3.0	19.5
" "	Green fruit	7.4	8.3	42.7	119.8
" "	Red fruit	—	7.5	—	122.8
" "	Stem	12.6	5.6	—	—
" "	Root	11.3	18.2	70.3	156.6
" "	Corolla	0.6	0.2	6.7	—
" "	None (check)	91.7	43.0	192.7	421.5

**26–30 cowpea leaves were inoculated with each kind of inoculum.*
†Infective juice of CMV consisted of a 1 : 6 dilution of juice expressed from tobacco in phosphate buffer at pH 7.

Properties of the Inhibitor

(*a*) *The Effect of Heat on the Inhibitor*

Aliquots, of 6 ml. each, of the aqueous pepper extract were placed in thin-walled tubes and were immersed in constantly agitated water baths at temperatures of 22°, 50°, 60°, 70°, 80°, 90°, and 100° C., respectively. After immersion for 10 min. the tubes were cooled immediately in running tap water. Ten drops of infective juice of CMV were added to each aliquot after the heat treatment. The effect of temperature was studied in two different tests at pH levels 5 and 7. Each test solution was rubbed on both primary leaves of 12 cowpea plants.

The results presented in Table IV show that the inhibitive power of the pepper extract is thermolabile, being slightly reduced by an exposure to 70° C. for 10 min. It was substantially diminished at 80° C. and was completely destroyed by a 10 min. exposure at 90° and 100° C. The inhibitor was more rapidly destroyed at the lower than at the higher hydrogen ion concentration.

(*b*) *Resistance of the Inhibitor to Aging in vitro*

An extract of green pepper leaves was allowed to stand in a stoppered flask at room temperature. At the end of 0, 24, and 48 hr. periods the inhibitive capacity of the extract was determined by bio-assay methods previously described. It was found that the inhibitor had not lost any of its effectiveness at the end of 48 hr. Other tests showed that the inhibitory capacity of the extract was not appreciably diminished by storage at 2° C. for three weeks.

(*c*) *Resistance of the Inhibitor to Drying in vitro*

Aliquots of the pepper macerate were allowed to evaporate to dryness in 24 hr. at a temperature of 30° C. The capacity of the residue to inhibit infection was determined at the end of 24-, 48-, and 72-hr. and 2-week periods. The original volume of the extract was restored by adding distilled water to

TABLE IV

THE EFFECT OF HEAT ON THE INHIBITIVE PROPERTY OF PEPPER EXTRACT TO CMV

Extract exposure temperature (° C.)	Average no. of lesions per inoculated cowpea leaf*	
	pH 5	pH 7
100	330.0	98.0
90	311.0	100.0
80	327.0	15.3
70	16.8	3.0
60	3.0	0.2
50	1.2	—
22	2.9	0.3
22 (Check, no pepper extract)	324.0	98.3

**The inoculum potential was not the same at the two pH levels.*

the residue. Virus preparations were then adulterated with the desired amount of the macerate and bio-assays were made. Drying for 72 hr. did not impair the effectiveness of the inhibitor, and desiccation for two weeks diminished its effectiveness only slightly.

(*d*) *The Effect of Dialysis on the Inhibitor*

The pepper extract was placed in a cellophane bag and was dialyzed against frequently changed distilled water for 48 hr. The effectiveness of dialyzed extract was compared with that of a nondialyzed extract by adding a few drops of virus juice before inoculation of cowpea leaves. This treatment did not reduce the inhibitive capacity of the pepper extract.

Partial Purification of the Pepper Inhibitor

The findings of several of the afore-described experiments relating to the properties of the inhibitor in the pepper suggested that it was a proteinaceous substance. Therefore standard methods for isolation of proteins were employed in an attempt to isolate the inhibitor in the pepper extract. Precipitation with 95% ethanol and ammonium sulphate combined with dialysis against distilled water yielded active preparations. The following procedure, which was a part of the method employed by Kassanis and Kleczkowski (9) for isolation of a virus-inhibiting protein from *Phytolacca*, was used: 30 ml. of 95% ethyl alcohol was added to 250 ml. of sap expressed from leaves and fruits of pepper plants, and the resulting coarse precipitate was removed by centrifugation. To the clear brown supernatant liquid 2 volumes of 95% ethyl alcohol were added and the precipitate that contains the inhibitor was centrifuged down. The precipitate was suspended in 30 ml. of 2% NaCl and the undissolved material was removed by centrifugation. The grayish supernatant liquid was dialyzed in a cellophane sack for 48 hr. against frequently changed distilled water. This liquid gave a positive biuret test and it exhibited considerable inhibitory power as demonstrated by bio-assays on cowpea.

However, each step in this isolation procedure resulted in some loss of activity of the inhibitor. Attempts to purify the active substance further by adsorption on celite at pH 7 and elution by a 10% NaCl solution yielded a preparation of low activity that showed a positive but weak biuret reaction. It was also found that prolonging the exposure of the inhibitor to ethanol from 20 min. to 5 hr. during the purification procedure diminished its inhibitory power by 65%.

Several tests were conducted to compare the activity of the alcoholic preparation with that of crude pepper sap. Aqueous solutions containing 10% of either the crude pepper sap or the extract purified by alcohol precipitation were applied to the upper and lower leaf surfaces of cowpeas before inoculation. In addition, comparisons were made with virus inoculum to which inhibitive preparations were added.

As can be seen from the results presented in Table V the alcohol-precipitate preparation displayed a much weaker inhibitive action than the crude sap. As observed in previous experiments, cowpeas varied appreciably in their response to the presence of the inhibitor from experiment to experiment.

An inhibitive preparation that was from three to five times as effective as that of the alcoholic-precipitate preparation, and only slightly less active than the crude pepper sap, was obtained by precipitation with ammonium sulphate. This procedure may be described as follows: saturated $(NH_4)_2SO_4$ was added to the supernatant pepper extract after the coarse cellular material was removed by centrifugation. At half saturation the inhibitor began to be precipitable, and a progressively heavier precipitate was formed as the concentration of the salt was increased. The precipitated inhibitor dissolved readily in water, and the salt was removed by dialysis against frequently changed distilled water for 48 hr. The capacity of this preparation to wet the leaf surfaces of cowpea and *Chenopodium* was almost equivalent to that of the crude pepper extract and was much superior to that of the alcoholic preparation.

TABLE V

THE COMPARATIVE EFFECTIVENESS OF CRUDE PEPPER SAP AND ETHANOL-PRECIPITATE PREPARATION IN INHIBITING LESION FORMATION BY CMV ON COWPEA

	Treatment of inoculated leaves						
	Crude extract with inoculum	Alcohol ppt. prep'n. with inoculum	Crude extract on upper leaf surface	Alcohol ppt. prep'n. on upper leaf surface	Crude extract on lower leaf surface	Alcohol ppt. prep'n. on lower leaf surface	Virus inoculum only
Experiment	Av. no. of lesions per inoculated leaf						
1	0.2	14.7	2.4	15.8			923
2	0.1	6.5	2.5	20.0			760
3	2.8	33.3			13.8	268.0	1480
4	0.1	0.7			2.0	21.6	373

Since crude pepper sap showed much greater surface-wetting properties than the alcohol-precipitate preparation, and since olein, palmitin, and stearin are contained in the fixed oil component of *Capsicum* (6), traces of these substances were added to the alcohol-precipitate preparation. These amended extracts were compared with crude sap for inhibitory power. These tests revealed that the inhibitory power of the alcohol-precipitate preparation was not increased by such amendments.

Studies of the Mechanism of Inhibition

The Effect of Separate Application of the Inhibitor and the Virus

During some exploratory tests it was learned that lesions developed on cowpea only when the virus preparations were applied soon after the abrasions were made in the leaves by the carborundum particles. These findings were interpreted to mean that the sites of virus entry in cowpea made by the abrasive remain receptive for only a short period of time. Also, when leaves were rubbed twice with an abrasive, the damage caused by the first rubbing interfered with the later infection response. Thus, for these experiments the technique involved applying the test solutions to the leaves before and after inoculation either by spraying or with a camel's-hair brush. After the inhibitive solutions were applied to the leaf surface they were blotted dry before inoculations were made. The test solution consisted of a 10% aqueous pepper extract.

The results presented in Table VI show that the application of the inhibitor to the cowpea leaves before inoculation was almost as effective in preventing infection as when a smaller amount was incorporated in the virus solution. In contrast, the pepper extract effected only a slight inhibitive response when applied after inoculation. Other preinoculation treatments in which the pepper extract was rinsed off with tap water before it had an opportunity to dry on the leaf surface to any appreciable extent also caused almost complete inhibition.

Additional experiments were conducted in which the inhibitor was applied to the lower surface of cowpea leaves by spraying or with a camel's-hair brush both either before or after inoculation.

TABLE VI

THE EFFECT OF APPLYING PEPPER EXTRACT TO THE UPPER SURFACES OF COWPEA LEAVES BEFORE AND AFTER INOCULATION WITH CMV

Time of application of inhibitor	Av. no. of lesions per inoculated leaf
24 hr. before inoculation	4.3
1 hr. before inoculation	2.6
Untreated	364.0
With virus inoculum	0.3
1 hr. after inoculation	276.0
2 hr. after inoculation	305.0

TABLE VII

THE EFFECT OF APPLYING PEPPER EXTRACT TO THE LOWER SURFACES OF COWPEA LEAVES BEFORE AND AFTER INOCULATION WITH CMV

Time of application of inhibitor	No. of lesions per inoculated leaf
18 hr. before inoculation	3.5
1 hr. before inoculation	3.2
15 min. before inoculation	4.6
With virus inoculum	0.3
15 min. after inoculation	201.0
Virus inoculum only (check)	224.0

The results presented in Table VII show clearly that lesion production was almost as much reduced when the inhibitive extract was applied to the lower leaf surfaces before inoculation as when it was added to the virus inoculum. Since postinoculation treatment does not reduce lesion production, in contrast to preinoculation treatment, apparently the inhibitor affects the first step of infection. Considerable variability in the response from plant to plant in the same experiment and between experiments was noted in these tests. This variability was interpreted to mean that the physiologic state of the host profoundly influences the inhibitive effect.

It was also observed that when the pepper extract was applied to one half of the lower leaf surface lesion production was reduced to the same extent on both halves of the upper inoculated surfaces. Moreover, on cowpea it was observed that the application of pepper extract to the surface of one primary leaf reduced to half the number of lesions produced on the opposite primary leaf. The results in this test are summarized in Table VIII.

The application of the pepper extract to the lower surfaces of leaves of *C. hybridum* before inoculation with CMV affected the production of local lesions much as it did on cowpea. Lesions produced on the untreated and treated inoculated leaves were in the ratio of 13 to 1. The application of the inhibitor to half-leaf portions exerted an inhibitive effect over the entire leaf.

Saturation of the soil with aqueous pepper extract 24 hr. before inoculation did not reduce the production of lesions on the leaves of cowpea plants.

The Effect of Pepper Extract on the Respiration of Cowpea Leaves

Since it was obvious that the pepper extract inhibited infection by its effect on the host plant rather than on the virus, it seemed worthwhile to

TABLE VIII

THE EFFECT OF APPLYING AQUEOUS PEPPER EXTRACT TO THE LOWER SURFACE OF ONE PRIMARY LEAF ON THE PRODUCTION OF LESIONS BY CMV ON THE OPPOSITE PRIMARY LEAF IN COWPEA

Treatment before inoculation	Av. no. of lesions per inoculated leaf
Pepper extract applied to lower surface of inoculated leaf	2.9*
No treatment of inoculated leaf, but pepper extract applied to lower surface of opposite primary leaf	37.0*
Check (water applied to lower surface of inoculated leaf)	76.0

**These differences were highly significant from the check as revealed by Student's t test.*

determine its effect on the metabolic activity of cowpea leaves as reflected by the change in the respiration rate. The direct method of Warburg (14) was used for measuring the oxygen uptake. In each case seven disks from cowpea leaves 1 cm. in diameter were placed in each Warburg vessel and the respiration was measured over a five hour period at 20° C. The CO_2 produced during the experiment was absorbed by the filter paper immersed in the 20% KOH solution in the center well of the reaction vessel. When the oxygen uptake of disks from leaves whose lower surfaces were treated with an 8% aqueous pepper extract was compared with that of disks from untreated leaves, no differences were observed. However, the oxygen uptake of leaf disks floated in 8% aqueous pepper extracts increased 40–50% over that of disks floated in water. This increase was shown in the first hour, and it was maintained for the entire five-hour period during which the experiment was conducted. In comparison, leaf disks floated in tobacco extracts showed only an 8–12% increase over those in water. When the leaf disks were removed from the Warburg reaction vessels those in pepper extracts showed evidence of a greater penetration of tissues by the liquid than did those in water. Apparently, the pepper juice increased the permeability of the cells. Tobacco juice induced a similar but less pronounced effect.

The Comparative Effect of Pepper Extract on the Respiration Rate of Leaf Disks of Cowpea, Datura, and N. glutinosa

Since pepper extract inhibited the production of lesions by the cucumber ringspot virus on cowpea, but not on either *Datura* or *N. glutinosa*, it seemed worthwhile to determine the effect of pepper extract on the respiration of leaf disks from these three hosts. Such an experiment was conducted.

It was found that the pepper extract induced a similar increase in the respiration rate of leaf disks of the three hosts. Also, the leaf disk tissue of these hosts showed a greater degree of both surface wetting and tissue penetration by the pepper extract than by water. In other experiments it was found that after the inhibitive property of the pepper extract was destroyed by heat it was still capable of inducing increased oxygen uptake in cowpea leaf disks equivalent to that of nonheated extracts. Obviously, the results of these respiration experiments do not indicate the mechanism whereby the pepper extract inhibits the formation of lesions on some hosts and not on others.

Discussion

From these results it is clear that sap from *Capsicum frutescens* L. contains a substance that inhibits virus infection of certain plants. Consequently, recognition of the fact that this inhibitor exists in pepper juice eliminates confusion in the identification, by means of indicator hosts, of viruses affecting this crop. Moreover, these findings point out that, although local lesion hosts may be used to identify viruses, use of them to estimate the concentration of virus particles in the source plant may lead to erroneous conclusions.

Interest in virus inhibitors has largely been focused on the mechanism of their action, and investigators have attempted to distinguish between those

that inhibit infection and those that inhibit virus increase. Since pepper extract is capable of reducing lesion formation on cowpea and *Chenopodium* when applied before the leaves are inoculated, and is unable to do so to any extent when applied after the leaves are inoculated, the inhibitor is considered to belong to the former group. In fact, all proteinaceous inhibitors that have been studied to date belong to this group.

Within recent years evidence has been accumulating to support the conclusion that inhibitors alter the susceptibility of the host rather than affect the virus particles directly. The inhibition brought about by the application of the pepper juice to the lower leaf surfaces of cowpea and *Chenopodium* is the most convincing evidence obtained in these studies to support this conclusion. Also, when an inhibitive response was produced on a host by pepper juice with one virus, a similar response was obtained on that same host with other viruses that cause primary necrotic symptoms. Gendron and Kassanis (7) reported earlier that the extent to which inhibitors of infection decrease numbers of local lesions is independent of the virus used, and depends only on the species to which inoculations are made. Thus, the generalization can be made that the host is determinative so far as the presence or absence of the inhibitive response is concerned.

The fact that lesion formation on cowpea and *Chenopodium* is prevented by applying the inhibitor either to lower leaf surfaces, or to areas a few centimeters remote from the point of virus inoculation, might suggest the influence of a readily transportable inhibitive agent. However, the pepper inhibitor displays characteristics of a protein, including inability to pass through a cellophane membrane. It must be assumed therefore that the particle size would be too great to allow ready passage through a protoplasmic membrane. If the protein is not itself highly mobile, its ability to act at a distance might be explained by the supposition that it is a conjugate protein whose reactive component is selectively absorbed at the leaf and cell surfaces, and subsequently moves to effective sites to block virus establishment and multiplication. Alternatively, the protein itself may effect at the epidermal surface a type of chain reaction which alters the cell metabolism so that protein synthesis is diverted from virus formation. An analogy might be drawn between this phenomenon and the report by French and Siminovitch (5) that *Escherichia coli* bacteria after exposure to T2 phage ghosts (viral protein) synthesize little protein during a refractory period when they resist infection with T2 bacteriophage.

To what extent transmission of viruses by vectors is affected by natural inhibitors is not definitely known. Doolittle and Walker (3), using aphids as vectors, transmitted cucumber mosaic virus from infected pokeweed to healthy cucumber plants, although they and others failed to show mechanical transmission. On the basis of these findings and those of a few other workers it is generally assumed that the capacity of aphids to transmit is not greatly influenced by the presence of these substances in source plants. On the contrary, some support for believing that vectors may be influenced by

inhibitors in source plants is suggested by Simons' (13) recent findings that in the transmission of southern cucumber mosaic virus by the green peach aphid (*Myzus persicae* Sulz.) chard was a much better virus source plant than pepper. Although other plausible explanations may be offered to explain the low efficiency of pepper as a source plant, there is justification for speculating that the aphids transfer the pepper protein inhibitor, which in turn reduces the susceptibility of the test host. Since inhibitor and virus are protein components of infected pepper plants, and presumably of the same cells, unless aphids are highly selective feeders it might be assumed that both proteins would be injected together into the host. Then, unless the inhibitor is altered by the aphid it might be expected to reduce infection substantially in certain plants. If this is true, aphid vectors should transmit tobacco etch and cucumber mosaic viruses more readily from tobacco to tobacco than from pepper to tobacco and perhaps other plants. If this could be demonstrated, it would lend support to the hypothesis that the transmission of many viruses by aphids is a mechanical process.

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THE ENZYMICALLY CATALYZED OXIDATION OF INDOLEACETIC ACID[1]

By E. R. Waygood, Ann Oaks, and G. A. Maclachlan

Abstract

Dialyzed wheat leaf extracts, catalase, and horse-radish peroxidase catalyze the decarboxylation and oxidation of indoleacetic acid at pH 5.0–6.0 in the presence of critical concentrations of manganese and monohydric phenols or resorcinol. The equivalent of 1 mole of carbon dioxide is liberated and 1 mole of oxygen consumed per mole of substrate. Manganic ions formed by a phenol–peroxidase–peroxide system initiate the decarboxylation and oxidation. A naturally occurring ether soluble factor from wheat leaves, and maleic hydrazide, can substitute for the active phenols. Catechol, hydroquinone, pyrogallol, scopoletin, and riboflavin, etc. competitively inhibit the oxidation. The nature of the active peroxide is discussed and a reaction sequence involving an organic peroxide or radical rather than hydrogen peroxide is submitted as being a possibility.

Introduction

Catalysis of indoleacetic acid oxidation by plant extracts has been the subject of considerable investigation and speculation during the past decade. Unfortunately, elucidation of the problem has been made more difficult by uncertainty as to the identity of the end product and the role of hydrogen peroxide, peroxidase, and flavoprotein in the reaction sequence.

Recently, the present authors (27) have attempted to reconcile some of the conflicting evidence associated with this complicated oxidation. The experimental evidence indicated that hydrogen peroxide produced during the oxidation was diverted to the oxidation of manganese by the Kenten–Mann system (16), which involves a monohydric phenol or resorcinol and peroxidase. The manganic ions so formed initiated the decarboxylation of the molecule which subsequently was oxidized aerobically. It was accepted that hydrogen peroxide was produced as a result of a flavoprotein-catalyzed aerobic oxidation previously postulated by Galston *et al.* (7).

Maclachlan and Waygood (20) have provided supporting evidence that indoleacetic acid oxidation is initiated by manganic ions. They have shown that indoleacetic acid is unstable in the presence of a manganic complex having a redox potential greater than 0.7 volts at pH 6.0. Under these conditions carbon dioxide is liberated from the molecule in theoretical amounts and oxygen is consumed by an intermediate. No additional cofactors are required. If the reaction sequence is similar to that of the enzymically catalyzed reaction there appears to be no necessity for postulating participation by a flavoprotein component in the enzymic reaction. Neither could it be demonstrated that hydrogen peroxide is produced in the non-enzymic manganic-catalyzed reaction.

This paper presents in more detail the experimental evidence supporting the hypothesis that enzymically produced manganic ions serve to initiate the

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decarboxylation and oxidation of indoleacetic acid. The principle has withstood further experimental tests, although some amendments in detail have been made and complete clarification of the reaction sequence will have to await identification of the end-product. The participation of a flavoprotein and hydrogen peroxide are questions which are discussed, but yet remain to be answered unequivocally. Further investigations have been made on the kinetics of the enzymically catalyzed reaction and these will be published in a subsequent paper (21).

Methods and Materials

Preparation of Plant Extracts

Leaves of wheat, *Triticum compactum* Host. var. Little Club, were harvested after they had grown from 12 to 15 days in greenhouse soil. The leaves were ground with sharp sand in a cold mortar and the brei was squeezed through nylon remnants. The press juice was centrifuged at 20,000 *g* at 2° C. for 30 min. and dialyzed against distilled water in a rocking dialyzer at 4° C. for at least three days. The dialyzate was centrifuged as before and the supernatant stored at −15° C. Intermittent freezing and thawing during usage rendered visually clear preparations which contained little or no green material.

Extracts of 10-day-old etiolated epicotyls, roots, and cotyledons from *Pisum sativum* L. were prepared in the same manner.

Manometric Methods

The oxidative decarboxylation of indoleacetic acid was followed by measuring oxygen uptake and carbon dioxide evolution by the direct method of Warburg in standard respirometers. Flasks of volume about 20 cc. contained the components in a liquid volume of 3.0 ml. with 0.2 ml. 2*N* KOH in the central well. Unless otherwise stated the concentrations of the components were as follows. Enzyme preparation (0.5 ml., 0.20 mgm. protein N); 150 μM. orthophosphate pH 6.0; 3 μM. manganese chloride; 1.5 μM. resorcinol or monohydric phenol; 6.67 μM. indoleacetic acid, dissolved in and made to pH 6.0 with *N* NaOH.

Colorimetric Methods

Indoleacetic acid destruction was also determined colorimetrically by using the Salkowski reagent in a procedure described by Gordon and Weber (11). One- or two-tenths milliliter of the contents of a Warburg flask or appropriate control was added to enough water to make up a volume of 2 ml. Three milliliters of the Salkowski reagent was added and the precipitate centrifuged off. After 30 min. the % transmission was read in a Coleman Universal Spectrophotometer at 520 mμ.

Maleic hydrazide, MH-40, and Agristrep 27% were supplied by T. Brian Harrison, Dominion Experimental Station, Harrow, Ontario, and pure

preparations of these were donated by the Naugatuck Chemical Co., Elmira, Ontario, and Merck and Co., Montreal, Quebec, respectively. Scopoletin was generously donated by Dr. W. A. Andreae, Science Service Laboratories, London, Ontario.

Experimental

Cofactor Requirements

Oxygen Uptake

When indoleacetic acid was added to a dialyzed wheat leaf preparation in orthophosphate buffer at pH 6.0 no destruction of the acid took place. If, however, manganous ions were present, partial destruction occurred with the uptake and release of an equivalent volume of oxygen and carbon dioxide. If in addition to manganous ions, a monohydric phenol or resorcinol were present, indoleacetic acid was completely destroyed, with the concomitant uptake and release of 1 mole each of oxygen and carbon dioxide per mole of indoleacetic acid. The oxidation was thermolabile, no carbon dioxide was released in a nitrogen atmosphere and in neither of these cases was indoleacetic acid converted to a non-Salkowski reacting substance (Table I). The gaseous exchange of the complete system as well as the oxygen uptake data for the system in the absence of one or more of the components is shown in Fig. 1.

A characteristic feature of the progress curve of oxygen uptake of systems catalyzed by 16 to 24 hr. dialyzed preparations from summer grown wheat leaves was a lag period lasting for 10 to 20 min. Extracts from winter grown leaves gave a lag period of only two to eight minutes. In spite of extended dialysis we have never been able to overcome the lag period completely.

The presence of manganese and monohydric phenols or resorcinol were essential for complete oxidation. Peroxidase was present in these preparations and Kenten and Mann (16) have shown that under such conditions in the presence of hydrogen peroxide manganese can be oxidized. The experimental results described in this paper show that manganese is oxidized and that manganic ions are essential for the subsequent decarboxylation and oxidation of indoleacetic acid.

TABLE I

EFFECT OF MANGANESE AND RESORCINOL ON THE OXIDATION OF INDOLEACETIC ACID (IAA)

Components	IAA destroyed, %	Gaseous exchange	
		μl. O_2	μl. CO_2
Enzyme alone	0	0	0
Mn^{++}	10	20–25	—
Resorcinol	15–20	30–40	—
Mn^{++} plus resorcinol	95–100	150–160	150–160
Mn^{++} plus resorcinol (anaerobic)	0	0	0
Mn^{++} plus resorcinol (boiled enzyme)	0	0	0

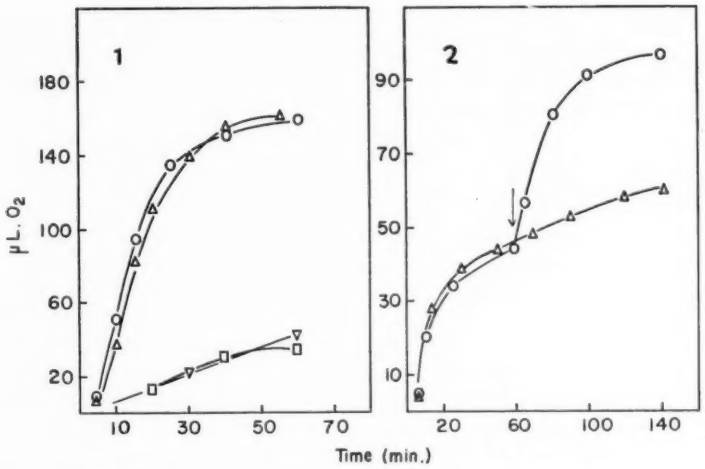

FIG. 1. Effect of cofactors on indoleacetic acid oxidation.
△——△ Oxygen uptake; ○——○ carbon dioxide evolution of complete system. Oxygen uptake ▽——▽ without manganese; □——□ without resorcinol. Wheat leaf enzyme from winter grown leaves. 158μl. indoleacetic acid.

FIG. 2. Inactivation of wheat leaf enzyme during indoleacetic acid oxidation.
○——○ Additional enzyme added at arrow. △——△ Additional substrate added at arrow.

Liberation of Carbon Dioxide

Although it was reported previously that carbon dioxide was evolved without any lag period in contrast to oxygen uptake, later experiments showed that this was not necessarily true. There was invariably a slight, often negligible lag period, but carbon dioxide was liberated always prior to the onset of oxygen consumption indicating that decarboxylation of the molecule precedes its aerobic oxidation. When the lag period of oxygen uptake was experimentally extended by the addition of catechol (Fig. 3) the lag in carbon dioxide evolution was extended also and preceded closely that of oxygen uptake. While the R.Q. of the reaction was generally close to unity, in some cases less carbon dioxide was evolved than oxygen consumed. The difference could be made up by tipping acid into the main compartment at the completion of the reaction. Hochster and Quastel (13) have shown that electron acceptance from biological systems by manganese dioxide in the presence of carbon dioxide results in the formation of insoluble manganese carbonate. The possibility that this reaction occurs in our system should not be excluded and it may play a part in determining the shape of the pH-activity curve at higher pH values (21).

Phenol Specificity

Kenten and Mann (16) have shown that only monohydric phenols and resorcinol are capable of mediating the oxidation of manganese by hydrogen peroxide and peroxidase. Similarly, only such phenols were effective in

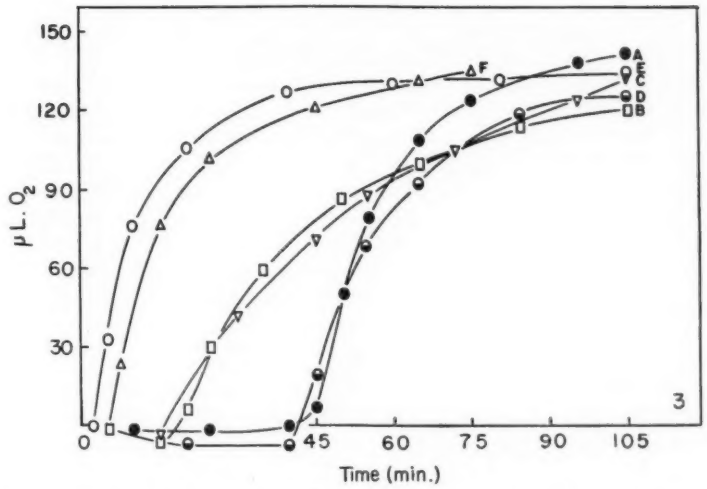

FIG. 3. The effect of oxygen, riboflavin phosphate, and white light on the catechol inhibited oxidation of indoleacetic acid (158 μl.).
○——○ (E) Control, complete system without catechol (air and darkness). ●——● (A) Control with 10^{-5} *M* catechol (air and darkness). ◒——◒ (D) Same as A in light (60 ft-candles). △——△ (F) Same as A in oxygen (manganese dioxide in air gave a similar progress curve). □——□ (B) Control with 4×10^{-5} *M* riboflavin phosphate (air and darkness). ▽——▽ (C) Same as B in light (60 ft-candles).

mediating the oxidation of indoleacetic acid by these wheat leaf extracts with manganese added (Table II). The optimum concentration for their activity is critical. Resorcinol shows greatest activity at 10^{-3} *M*; less activation occurs at lower or higher concentrations (21). The optimum concentration for dichlorophenol is 5×10^{-4} *M* (21). Dichlorophenol was most efficient since it shortened the lag period, and in the absence of extraneous manganese caused a greater oxidation of indoleacetic acid than the other phenolic compounds. Goldacre and Galston (9) have shown that dichlorophenol and related compounds inhibit catalase. This, they state, would have the effect of protecting hydrogen peroxide apparently required for indoleacetic acid oxidation. However, it is possible that dichlorophenol oxidizes manganese more efficiently than do the other phenols. The ability of these phenols to oxidize indoleacetic acid partially in the absence of added manganese may be due to their capacity to coordinate non-dialyzable manganese.

The inactivity of catechol, hydroquinone, and pyrogallol is directly correlated with their ability to reduce manganic ions instantaneously, a property which is not associated with the "active phenols" (Table II). To demonstrate this the various phenols were added dropwise to a manganiversene solution prepared as described previously (20). Decolorization of the red manganiversene was indicative of the reduction of manganic ions.

The affinity of catechol for manganic ions was shown strikingly by the presence of 5×10^{-6} *M* catechol in the complete system. The lag period

TABLE II

EFFECT OF VARIOUS PHENOLS ON THE OXIDATION OF INDOLEACETIC ACID AND THE REDUCTION OF MANGANIC IONS

Compound	Lag period, min.	Oxygen uptake, μl.	Decolorization of manganiversene in 15 min.
Catechol	—	8	Immediate
Hydroquinone	—	12	Immediate
Pyrogallol	—	5	Immediate
Resorcinol	10	150–160	Slow
o-Cresol	10	150–160	Slow
Phenol	10	150–160	Slower
2,4-Dichlorophenol	5	150–160	Slowest
Resorcinol and catechol (5×10^{-6} *M*)	174	140	—
Maleic hydrazide	10	150–160	None
Ether soluble factor*	20	150	Not tested

NOTE: *Theoretical oxygen equivalence for 1 mole = 158 μl.*
Concentration of all phenolic compounds = 1.5 μM./3.0 ml.
**Concentration unknown.*

was extended, in some experiments as much as three hours (Table II). This effect of catechol is delicately balanced and for a specific concentration the lag period may vary considerably (cf. Fig. 3). Its effect on the reaction is due to its competition with indoleacetic acid for manganic ions. Such reactions involving competition for manganic ions by endogenous factors may be considered responsible for the inherent lag period of the oxidation. It is noteworthy that extracts from summer grown wheat leaves show a greater lag period than those from winter grown wheat and the former were invariably characterized by the presence of one or more substances which were dialyzable with difficulty, were probably phenolic in nature, and colored the extracts brown during prolonged storage in the cold.

Other substances that replace the phenolic compounds are maleic hydrazide, and an ether soluble factor isolated from the dialyzing medium (see p. 919). Accordingly, these may be included in the category of peroxidase substrates the oxidation products of which are capable of oxidizing manganese. The data for these also are shown in Table II. The activity of maleic hydrazide increases with increasing concentration and this cofactor does not show an optimum concentration at which it is most effective (21).

The Participation of Peroxidase

The oxidation of manganese in the dark has been shown for systems containing hydrogen peroxide, peroxidase, and certain peroxidase substrates (16). The necessity for manganese and such peroxidase substrates in the oxidation of indoleacetic acid indicates that an enzyme having peroxidatic activity participates in the reaction. Undialyzed, but purified horse-radish peroxidase (Nutritional Biochemical Co.) catalyzed the oxidation without the prior addition of manganese and phenolic compound, but it required these cofactors after dialysis against versene (22).

At low concentrations of wheat leaf extract, indoleacetic acid was only partially oxidized and it was shown that under these conditions the peroxidatic activity of the extracts had been lost. Further oxidation could be brought about only by the addition of more enzyme, but not more substrate (Fig. 2). Kenten (15) found also that his peroxidase preparations from bean root were inactivated during the course of the reaction with indoleacetic acid. Inactivation appears to be caused by some intermediate produced during the oxidation. Complete oxidation of indoleacetic acid depends on the presence of optimum concentrations of manganese and phenolic compound as well as sufficient peroxidase. In cases where incomplete oxidation occurs in the presence of optimum concentrations of the cofactors this usually can be traced to limiting peroxidase activity caused by inactivation during storage, etc.

The Role of Manganese

Wagenknecht and Burris (25) first demonstrated that the addition of manganese stimulated the oxidation of indoleacetic acid catalyzed by dialyzed bean root and pea epicotyl extracts. Magnesium, cupric, zinc, and ferric ions did not substitute. We have confirmed their findings and also found that cobalt is inactive. The inactivity of the metal ions capable of forming redox systems is most probably due to their redox potentials being lower in complex than that of indoleacetic acid (E_0' = ca. 0.7 v. at pH 6.0) (20).

The Effect of Mangani-complexing Agents

The oxidation of indoleacetic acid was inhibited completely by the presence of either citrate or pyrophosphate at a concentration of 8.3×10^{-3} *M*. Both these substances with manganic ions form colored chelate complexes that have relatively low redox potentials at pH 6.0 (E_0' = 0.60 v.) (20). In contrast, the mangani-complex formed with ethylenediaminetetracetate (versene) shows a higher potential at this pH (E_0' = 0.710 v.) and versene had a tendency to augment the over-all oxygen uptake at the same concentration. With increasing concentration of versene there was some depression of the rate.

Kenten and Mann (17) have shown that hydrogen peroxide produced by aerobic dehydrogenase systems can be disposed of by oxidizing manganese to the trivalent stage via an appropriate phenol–peroxidase system. In orthophosphate, the rate and absolute amount of oxygen uptake of such a system is half of that in pyrophosphate, since in orthophosphate the manganic ions so formed by the utilization of one hydrogen peroxide molecule split another and themselves become reduced. On the other hand the mangani-complex formed in pyrophosphate splits hydrogen peroxide slowly owing to its weak ionization, and therefore it accumulates. Thus the doubling of oxygen uptake by pyrophosphate in such systems would afford a reliable test for hydrogen peroxide production and as well would indicate manganese oxidation and the non-requirement of the aerobic dehydrogenase for manganic ions. Since pyrophosphate completely inhibited the decarboxylation and oxidation of indoleacetic acid when catalyzed by the Kenten–Mann system it must be

concluded that manganic ions are essential. Citrate is capable also of forming a mangani-complex (16, 20) and thereby removes the essential manganic ions from the reaction site. Versene, on the other hand, forms a more readily dissociable complex and would compete with indoleacetic acid for manganic ions only at high concentrations.

The finding by Galston *et al.* (7) that manganous ions inhibited the oxidation of indoleacetic acid catalyzed by the oxidase from etiolated pea epicotyls can be explained by their use of citrate–phosphate buffers exclusively. Kenten (15) found that the presence of citrate or pyrophosphate completely overcame the activating effect of manganese on indoleacetic acid oxidation catalyzed by bean root extracts in orthophosphate. It is probable that indoleacetic acid was being oxidized directly by hydrogen peroxide in Galston's system. The presence of manganese and an endogenous factor capable of mediating its oxidation would divert peroxide from indoleacetic acid oxidation to the oxidation of manganese. The manganic ions so formed would be complexed and unavailable for indoleacetic acid oxidation.

There can be little doubt that the effect of manganese depends on the choice of buffer. The original reports of manganese activation came from investigators who used orthophosphate buffers solely (12, 25, 27).

The Lag Period

The Effect of Light

The inherent lag in oxygen uptake catalyzed by extracts from summer grown wheat leaves was invariably found in systems oxidizing in air in respirometer flasks shaking in the diffuse light of the bath. This was unchanged if the flasks were darkened. However, when the flasks and their contents were illuminated with white light of intensity 60 foot-candles, oxygen uptake occurred almost immediately after the indoleacetic acid was tipped in. Red light was ineffective, but blue light at the same intensity was as effective as white light (Table III).

TABLE III

EFFECT OF DIFFERENT WAVELENGTHS OF LIGHT ON THE LAG PERIOD

Kind of light	Lag period (min.)	
	By endogenous factors	Experimentally induced*
Dark	10	30
White	2	5
Blue	2	2
Red	10	35

**By natural factors.*

The effect of different wavelengths of light was demonstrated more clearly on a lag period experimentally induced by the presence of, instead of resorcinol, a small portion of the total ether soluble fraction from the dialyzing medium. This fraction contains a number of components (see p. 917) one of which substitutes for resorcinol whereas the others presumably compete with indoleacetic acid for manganic ions. The net effect was that the lag period was extended, in this case to 30 min., but oxidation eventually proceeded, though at a much slower rate. White and blue light diminished this lag period significantly whereas red light was again ineffective. The results are shown in Table III.

Galston *et al.* (7) found that white and blue light reversed the inhibition caused by manganese or catalase on their indoleacetic acid oxidase system from peas in orthophosphate–citrate buffer. Earlier, Galston (6) had proposed from studies on the phototropic action spectrum of oat coleoptiles, in which blue, but not red light is effective, that riboflavin must be the photoreceptor pigment in this phenomenon. Since he found that riboflavin also sensitized the photoinactivation of indoleacetic acid and the native diffusible auxin from oat coleoptiles and since light activation of the flavoprotein xanthine oxidase had been reported previously he proposed that one of the steps in the reaction sequence of the indoleacetic acid oxidase system was catalyzed by such a flavoprotein.

However, Kenten (15) has shown that indoleacetic acid oxidation can be catalyzed by bean root enzyme preparations from which the flavoprotein components have been rigorously removed. This, together with the demonstration that manganic ions per se can initiate the decarboxylation and oxidation of indoleacetic acid, appears to make it unnecessary to postulate that additional cofactors catalyze the oxidation, providing that the pathway is the same in all reactions.

Andreae (2) has shown also that light activated riboflavin can oxidize monohydric phenols and resorcinol, which in turn oxidize manganese non-enzymically. The production of manganic ions was demonstrated by complex formation with pyrophosphate at pH 7.0° and 0° C. Catalase, when added, increased the accumulation of manganipyrophosphate. It was explained that this disposed of hydrogen peroxide (preventing its reaction with manganic ions) produced by the aerobic restoration of the hydrogen acceptor riboflavin. If such a mechanism were operating in our illuminated systems, in which the lag period was lengthened by natural dialyzable factors (Table III), the extra production of manganic ions should not only increase the oxidation rate, but also raise the oxygen consumption to values that exceed the theoretical. However, under these conditions light appeared to exert its effect only on the lag period. The rate of oxygen uptake was not affected and although in a few cases the over-all oxygen uptake was increased about 5%, in as many cases, oxygen uptake was depressed to the same extent (cf. Fig. 3).

When the lag period was experimentally extended by the addition of 5×10^{-6} *M* catechol it was not overcome by illumination and the subsequent

rate of oxygen uptake was slightly depressed with little change in the over-all oxygen uptake. On the other hand the presence of riboflavin or its phosphate resulted in a marked decrease in the length of the catechol induced lag period and this was not altered significantly by illumination. Oxygen consumption however was retarded. The results of typical experiments are assembled in Fig. 3, which also includes the effect of oxygen on this lag period for comparison. Maclachlan and Waygood (22) have considered the possibility that riboflavin decreases the lag period by promoting formation of manganic ions, indirectly via resorcinol, but its reduced form competes with indoleacetic acid for manganic ions, thus retarding the reaction. The amount of inhibition would be dependent on the steady state equilibrium between the oxidized and reduced forms of riboflavin. In this connection it should be noted that in one of our earlier experiments the retardation of an otherwise uninhibited system by riboflavin phosphate was alleviated to some extent by illumination. As will be seen in the next section on the effect of oxygen, riboflavin appears to play a role in the production of manganic ions by the resorcinol 'oxidase' system. Although the evidence does not favor the direct participation of riboflavin or related compounds in the reaction sequence, it is highly probable that its presence *in vivo* could profoundly affect the metabolism of indoleacetic acid.*

The Effect of Oxygen

By replacement of the air in the reaction flasks with oxygen the lag period caused by naturally occurring dialyzable factors was overcome and the over-all oxygen consumption was increased to values that in some instances exceeded the theoretical oxygen equivalence. When the lag period was experimentally extended by the presence of 5×10^{-6} *M* catechol, oxygen overcame the lag period almost completely (Fig. 3), but the rate of oxygen uptake was not affected. This marked effect of oxygen is due to the increased production of manganic ions since manganese dioxide is equally effective in overcoming the lag period. It was observed that when oxygen was present the colored oxidation products of resorcinol began to form prior to the tipping in of the indoleacetic acid. Further experiments to be reported in detail later have shown that this aerobic oxidation of resorcinol probably involves the oxidation of manganese and may be catalyzed in darkness and air by wheat leaf extracts, horse-radish peroxidase, and catalase or to a lesser extent non-enzymically by riboflavin in an atmosphere of oxygen. Such an 'oxidase' type reaction occurring even to an almost negligible extent would serve to explain the initiation of indoleacetic acid oxidation by preformed manganic ions without having to assume the presence of endogenous hydrogen peroxide. The increased oxygen uptake which occurs in oxygen may be explained by the additional oxygen uptake caused by the oxidation of resorcinol. It would be

*We have since shown that riboflavin retards indoleacetic acid oxidation by operating as a chain transfer agent. Blue light overcomes its inhibitory effect (see Waygood, E. R. and G. A. Maclachlan. The effect of catalase, riboflavin and light on the oxidation of indoleacetic acid. Physiol. Plantarum, 9: 607-617. 1956).

safe to assume that both oxygen and light affect the oxidation of indoleacetic acid by accelerating the production of manganic ions via as yet unknown reactions involving phenolic compounds and perhaps riboflavin.

Effect of Manganese Dioxide

According to this hypothesis any potential oxidant of manganese should overcome the lag period to an extent dependent on the rate of oxidation. Manganese dioxide, which is potentially capable of oxidizing manganous ions (20), shortens the lag period caused by endogenous dialyzable factors, but does not abolish it completely. However, it is as effective as oxygen in overcoming the catechol induced lag period and the progress curve for the oxygen atmosphere shown in Fig. 3 is not significantly different from that for the system containing manganese dioxide in air. Manganiversene also overcomes the catechol-induced lag period and these effects indicate that catechol extends the lag period by competing successfully with indoleacetic acid for manganic ions. Once oxidized it has no further effect on the reaction.

Manganese dioxide is also capable of splitting hydrogen peroxide catalytically and if free hydrogen peroxide were produced and utilized in the reaction then manganese dioxide should inhibit. The fact that it does not suggests that either free hydrogen peroxide is not produced or that peroxide in complex at the reaction site is spatially separated from manganese dioxide.

Manganese dioxide did not itself substitute for manganese in the reaction; however, both together, but not separately, cause a slow destruction of indoleacetic acid in the absence of either a phenolic compound, or enzyme, or both, a fact which lends further support for the participation of an intermediate valence stage of manganese in the reaction.

Effect of Hydrogen Peroxide

The addition of hydrogen peroxide to the reaction system prior to the addition of indoleacetic acid abolishes the lag period. This is similar to its action in overcoming the lag period encountered in the manganese catalyzed oxidations of certain dicarboxylic acids (18). We have utilized the aldehyde oxidase system present in the enzyme preparations to provide a uniform and constant supply of hydrogen peroxide during indoleacetic acid oxidation. Acetaldehyde was allowed to volatilize from the side arms of Warburg flasks with the enzyme buffered by ortho- or pyro-phosphate at pH 6.0. Indoleacetic acid was placed in the opposing side arms and manganese and a phenol were present as required.

When manganese and resorcinol were present the rate of acetaldehyde oxidation in pyrophosphate buffer was twice that found when orthophosphate was used (Fig. 4). A priori, this indicates hydrogen peroxide production, presumably according to the following equation:

$$CH_3CHO + H_2O + O_2 \longrightarrow CH_3COOH + H_2O_2 .$$

Manganese was therefore not essential, but disposed of hydrogen peroxide as described previously (p. 911).

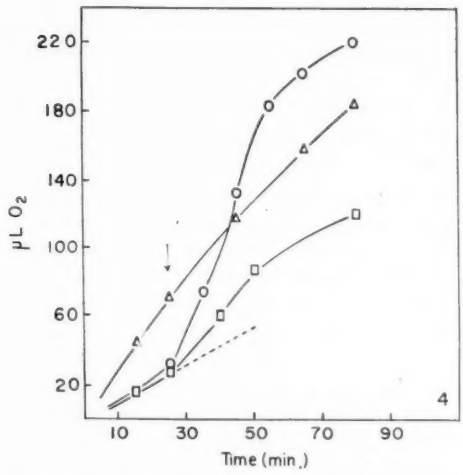

FIG. 4. Addition of indoleacetic acid during steady state oxidation of acetaldehyde by wheat leaf enzyme.
○——○ Orthophosphate buffer with manganese and resorcinol. △——△ Pyrophosphate buffer with manganese and resorcinol. □——□ Orthophosphate with manganese dioxide and manganese chloride. 158 μl. indoleacetic acid added at arrow.

When the reaction was proceeding in orthophosphate, the addition of indoleacetic acid caused an immediate and increased rate of oxygen uptake which levelled off to the former rate after the equivalent of 1 mole of oxygen per mole of indoleacetic acid added had been consumed (Fig. 4). If either manganese or resorcinol or both were absent no additional oxygen uptake occurred, although according to the Salkowski test 50% of the indoleacetic acid had been destroyed compared to about 100% in their presence. Under these conditions manganese dioxide in effect replaced resorcinol as oxidant of manganese and induced an additional oxygen uptake which showed an inflection point at a value equivalent to ½ mole per mole of indoleacetic acid (Fig. 4). The only reasonable explanation for the halving of the molar equivalence under these conditions is that an intermediate peroxide, being unable to react peroxidatically with resorcinol, is disposed of with oxygen release. The rapid oxidation in the absence of a phenolic compound can be explained by assuming that the formation of manganic ions from a mixture of manganese dioxide and manganous ions is augmented by the continual presence of hydrogen peroxide produced by acetaldehyde oxidation. Destruction of indoleacetic acid under these conditions amounted to 83% (27).

In pyrophosphate buffer, with both manganese and resorcinol present, no additional oxygen uptake occurred after the addition of indoleacetic acid (Fig. 4). Pyrophosphate interferes with the Salkowski test and accurate measurements of indoleacetic acid destruction could not be obtained.

In the absence of any one or more of the additional components essential for the oxidation of manganese and thus the complete destruction of indoleacetic acid, there was always approximately 50% destruction of indoleacetic acid (27). This destruction must be due to the direct oxidation of indoleacetic acid by hydrogen peroxide produced during the oxidation of acetaldehyde. The oxidation–reduction potential of the hydrogen peroxide – water system ($E_0 = 0.682$ v.) is very close to what is considered to be the potential of the indoleacetic acid system ($E_0 = 0.7$ v.) (20) and direct oxidation by hydrogen peroxide is highly probable. However we have never been able to show complete destruction by hydrogen peroxide under these conditions.

Effect of Catalase

Reactions in which hydrogen peroxide is essential should be inhibited completely by catalase unless it operates peroxidatically, or is inactivated during the course of the reaction. Catalase has been shown to inhibit photoreversibly the indoleacetic acid oxidase from etiolated pea epicotyls and horse-radish peroxidase and it was concluded that hydrogen peroxide was essential for the direct peroxidation of indoleacetic acid. Catalysis of indoleacetic acid oxidation by the Kenten–Mann system makes it unnecessary to postulate a direct hydrogen peroxide requirement, but the effect of catalase should be similar if free hydrogen peroxide is both produced and utilized.

Our previous report that catalase extends the lag period in the same manner as its effect on the oxidation of certain dicarboxylic acids has been shown to be due to the presence of endogenous dialyzable factors (giving the catechol effect) in the preparations of beef liver catalase (Nutritional Biochemical Co.). Well dialyzed catalase preparations have no effect on the oxidation. Indeed they catalyze the oxidation of indoleacetic acid in the presence of manganese and resorcinol or preferably 2,4-dichlorophenol. Catalysis causes an inactivation of the peroxidatic activity of catalase, but not of its catalatic activity. These experiments will be reported later (see footnote p. 914).

Andreae (2) found that catalase increased the accumulation of manganic ions by his light activated riboflavin–phenol system. His explanation that it does so by disposing of hydrogen peroxide, which would react with and decrease the accumulation of manganic ions, is not consistent with the experimental facts that manganipyrophosphate reacts only slowly with hydrogen peroxide. Thus Kenten and Mann (17) were able to show a doubling of oxygen uptake in systems producing hydrogen peroxide because of this property. It is highly probable that the catalase preparation used by Andreae (2) was acting peroxidatically.

Effect of Components of the Dialyzing Medium

In preliminary experiments it was shown that 2-cm. squares of filter paper, each containing one of 13 chromatographically separated components of the alcohol and water-soluble–ether-insoluble fraction of wheat leaves, completely inhibited oxygen uptake by indoleacetic acid catalyzed by the manganese–

resorcinol–peroxidase system. Since dialysis removed inhibitory factors, attempts were made to isolate these from the dialyzing medium and to determine if any natural factors were present that could substitute for resorcinol in the system.

Crude wheat leaf extract (140 ml.) was dialyzed for 24 hr. against three changes of distilled water each comprising a volume of 1 liter. The first 2 liters were combined and concentrated *in vacuo* at 40°–50° C. to a volume of 32 ml. The concentrate, pH 4.5, was extracted twice with an equal volume of ether and both the ether-soluble and ether-insoluble fractions were allowed to evaporate to dryness at room temperature after which they were dissolved in 5 ml. of water.

The results of an experiment in which various concentrations of both fractions were added to the system in the presence and absence of resorcinol are shown in Table IV. The data show that both fractions contain inhibitory factors that extend the lag period, but are apparently destroyed (like catechol) prior to oxygen uptake. The fact that oxygen uptake occurs in the absence of resorcinol indicates the presence of a component in both fractions that can substitute for resorcinol. Owing to the slower rate of production of manganic ions in the system without resorcinol, the inhibitory factors are not as rapidly destroyed as in its presence. This results in a slower rate of oxygen uptake as evidenced by the time taken to reach equilibrium (Table IV). The extended lag period induced by the ether soluble fraction was used in experiments to determine the effect of different wavelengths of light described previously, and the results in Table III show that this lag was greatly decreased by white and blue light, but not red light.

The ether-soluble fraction was subsequently separated chromatographically in butanol – water – acetic acid (4 : 5 : 1). Ferric chloride spray showed blue phenolic spots at R_f 0.86 and the frontier, and two white spots at R_f 0.08 and 0.28. Fluorescent spots were observed at the frontier and R_f 0.31. The spots

TABLE IV

EFFECT OF COMPONENTS OF THE DIALYZING FLUID

Fraction	Lag period, min.	Oxygen uptake, μl.	Time to attain equilibrium, min.
Control	10–15	140	60
Ether-soluble			
Undiluted	15	26	160 plus
Diluted 10-fold	60	110	150
" 50-fold	10	140	100
" without resorcinol	30	90	280 plus
Ether-insoluble			
Diluted 50 fold	60	140	160
" without resorcinol	30	90	280 plus

NOTE: *All additions were of 0.1 ml. volume.*

were cut out on 2-cm. squares of paper and each placed in the main compartment of a respirometer flask containing the complete system. The results are shown in Fig. 5. In all cases the lag period was prolonged and its length appeared to be related to the R_f value of the component, although this could well be a concentration effect. The trigger-like bursts of oxygen uptake indicated that they all exhibited the catechol effect and presumably competed for and were destroyed by manganic ions.

The region showing blue fluorescence with ultraviolet at the frontier was isolated from larger scale chromatograms and the components were eluted from the paper with phosphate buffer. At the time, the solution was tested in the glucose-6-phosphate-TPNH oxidase system mediated by manganese and resorcinol (22) and it was found to substitute effectively for resorcinol. Our recent isolations of this factor have been made by acidifying the concentrated dialyzing medium with phosphoric acid and then extracting with ether. This ether soluble fraction, without further purification, substitutes for resorcinol and mediates the oxidation of indoleacetic acid, although it contains catechol type competitive inhibitors which produce the characteristic effect at higher concentrations. Kinetic studies of the natural factor are reported in a later paper (21).

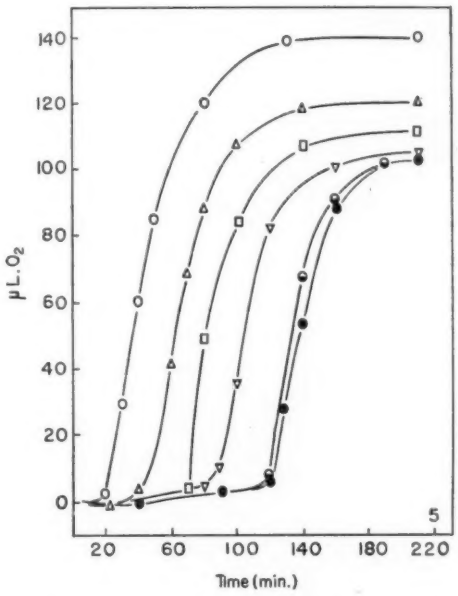

FIG. 5. The effect of chromatogram spots from the ether soluble fraction on indoleacetic acid oxidation.

○—○ Control. △—△ R_f 1.0. □—□ R_f 0.86. ▽—▽ R_f 0.31. ◒—◒ R_f 0.28. ●—● R_f 0.08. 158 μl. indoleacetic acid.

Miscellaneous

Effect of Other Growth-regulating Substances

Dialyzed extracts of wheat leaves fortified with manganese and resorcinol were unable to catalyze the aerobic oxidation of either indolepropionic or indolebutyric acids. Since manganic ions per se catalyze the decarboxylation and oxidation of these acids to a certain extent (14) it may be that their oxidation initiated by manganic ions does not lead to the formation of peroxide upon which the continual oxidation of manganese appears to be dependent in the enzymically catalyzed reaction. They may also require a higher concentration of manganic ions to initiate decarboxylation than that produced enzymically.

It has been shown that other plant extracts as well as horse-radish peroxidase are capable of catalyzing the partial oxidation of indolebutyric and especially indolepropionic acid (14, 25). These oxidations were stimulated by manganese, although the presence of monohydric phenols did not appear to be essential. An 'oxidase' type of oxidation has been suggested by Kenten (14) for the oxidation of indolepropionic acid by horse-radish peroxidase. However, the possibility of endogenous phenols and manganese being present in the enzyme preparation was not excluded by his method of purification. The oxidation of indolepropionic acid was all the more remarkable since it was not accompanied by the liberation of carbon dioxide. This was in direct contrast to the manganic-catalyzed oxidation of indolepropionic acid in which always more carbon dioxide was liberated than oxygen consumed (20). While skatole was actively oxidized by mangani-versene (20) it was only partially oxidized enzymically. However, the addition of skatole to a system actively oxidizing indoleacetic acid increased the over-all oxygen uptake to a value greater than the molar equivalence of indoleacetic acid. It was concluded that a potential surplus of manganic ions was available during indoleacetic acid oxidation.

Our dialyzed wheat leaf extracts were also incapable of catalyzing the aerobic oxidation of 2,4-dichlorophenoxyacetic acid or naphthaleneacetic acid, neither could it be shown that the former contained some 'active' 2,4-dichlorophenol (cf. 10).

Activators and Inhibitors

Mention has been made previously of the activating effect of oxygen, manganese dioxide, and in certain cases light. These apparently operated by accelerating the production of manganic ions. A natural factor and maleic hydrazide activated the oxidation by replacing the phenolic compounds, e.g. resorcinol, as oxidants of manganese.

Enzyme poisons such as cyanide, azide, hydroxylamine completely inhibited the reaction at 10^{-2} *M* presumably by their effect on peroxidase. Semicarbazide and hydrazine sulphate inhibit at the same concentration.

There appear to be at least three types of substances each producing a different form of competitive inhibition. The first comprises the catechol types, which prolong the lag period by competing for and being destroyed by manganic ions. Once destroyed they have no further effect. Substances

coming within this category are catechol, pyrogallol, phloridzin, rutin, and all are capable of extending the lag period from 20 to 30 min. at a concentration of about 10^{-5} *M*.

Citrate and pyrophosphate completely inhibit the reaction above a concentration of 0.83×10^{-3} *M*. Below this concentration they extend the lag period and retard the reaction. However, their destruction is probably caused by an agency other than manganic ions since citrate and pyrophosphate are stable in manganic complex and prior incubation of these substances with the enzyme preparation partially overcomes their effect.

The third group, of the hydroquinone type, do not extend the lag period, but retard the reaction at the outset. This group is exemplified by hydroquinone, *p*-benzoquinone, scopoletin, dinitrophenol, and riboflavin. It is considered that these substances retard the reaction by competing for manganic ions, but their oxidized forms cause a further production of manganic ions indirectly. Thus the amount of inhibition is dependent on the steady state equilibrium between their oxidized and reduced forms.

Agristrep (27% Merck and Company) at a concentration of 0.83×10^{-3} *M* streptomycin inhibits the oxidation 50% but pure streptomycin had no effect on the reaction, and the inhibiting principle of Agristrep must be due to some other component of this preparation.

The Formation of Hydrogen Peroxide

The demonstration by Maclachlan and Waygood (20) that manganic ions initiate the decarboxylation and oxidation of indoleacetic acid in the absence of additional cofactors and without the apparent production of hydrogen peroxide raises doubts concerning the participation of free hydrogen peroxide in the enzymically catalyzed oxidation. The stable yellow final product produced by the enzymically catalyzed reaction appears to be identical with that produced non-enzymically, although neither has been identified as yet. If such is the case the intermediate arising from the primary reaction with manganic ions is oxidized directly by molecular oxygen. The intermediate could be an organic peroxide that enters into a peroxidatic reaction with the phenolic compound in the enzymically catalyzed reaction and no free hydrogen peroxide need be produced. Neither catalase nor manganese dioxide nor ferric ion, all of which catalytically split hydrogen peroxide, inhibited our reaction, and the peroxidatic activity of catalase could also involve an organic peroxide rather than free hydrogen peroxide. The most convincing evidence for hydrogen peroxide production has been shown by Andreae and Andreae (2, 3), who observed the quenching of fluorescence of scopoletin during indoleacetic acid oxidation, a very sensitive test for hydrogen peroxide (1). However, doubts can be raised regarding this evidence since the reactions of manganic ions and hydrogen peroxide are so often similar. Manganiversene oxidizes scopoletin, but not manganicitrate, and Andreae (2) used citrate as buffer. Accordingly the conclusions of Andreae (2) are probably correct for the system he used, although the possibility of the quenching of the fluorescence of scopoletin by an organic peroxide should not be excluded.

Discussion

The results of the experiments described in this paper provide additional evidence in support of the hypothesis previously put forward (27) that decarboxylation and oxidation of indoleacetic acid is initiated by manganic ions enzymically produced by a phenol–peroxidase–peroxide system similar to that described by Kenten and Mann (17, 18).

In analogy to other metal ion-catalyzed oxidations (28) it is probable that the manganic ion, by placing a high positive charge on the carboxyl group of indoleacetic acid, withdraws an electron and a hydrogen ion, thereby releasing carbon dioxide. The product of this primary reaction is most probably a free radical intermediate since in the non-enzymically catalyzed reaction it can either polymerize or consume oxygen depending on its rate of production (20). In the enzymically catalyzed reaction polymerization does not occur, but oxygen is consumed and at pH 6.0 a stable yellow product is formed similar to that produced in the non-enzymically catalyzed reaction (20).

It has not yet been established whether oxygen reacts with an intermediate hydrogen acceptor to produce hydrogen peroxide. Participation by a flavoprotein component has been postulated and was accepted by the present authors previously (27); however, the results of these and other investigations (15), while not excluding it, make this less of a possibility than was thought formerly. Moreover, whereas the evidence would appear to favor hydrogen peroxide formation and utilization, reasonable doubts can be raised regarding the validity of this evidence. An alternative possibility is that the free radical intermediate reacts with oxygen and forms an organic peroxide or radical perhaps of the type suggested by Ray and Thimann (23). Conceivably, such a peroxide or radical could react with the phenolic compound in a peroxidase-catalyzed reaction withdrawing an electron and hydrogen ion to produce an aryl radical capable of oxidizing manganese. Or on the other hand the organic peroxide or radical may react with water in the same way to produce an hydroxyl radical that subsequently would enter into the peroxidase-catalyzed oxidation of the phenol. In either case the end product would be the same and its empirical formula on paper would resemble that postulated by Ray and Thimann (23).

The reaction would be started by manganic ions initiated by a relatively slow oxygen-dependent 'oxidase' type of reaction involving an appropriate phenol, e.g. resorcinol. Once the reaction was initiated the peroxide produced as a result of the oxidation of indoleacetic acid would oxidize the phenolic compound more efficiently than the 'oxidase' reaction. It is highly probable that monophenols and resorcinol, which are incapable of forming stable quinones, would form highly reactive intermediate hydroperoxides (5) and/or semiquinol free radicals (26) capable of oxidizing manganese and at the same time reforming the phenolic compound. It should be noted that at the completion of oxidation most of the resorcinol is in some oxidized form. This could only come about if there were at least two reactions potentially capable

of producing manganic ions for one utilizing them, thus allowing a phenolic radical to dimerize. There is some evidence of a potentially available surplus of manganic ions, but no surplus would exist over and above the stoichiometric demand made on the system by the primary reaction of indoleacetic acid with manganic ions. Thus the free radical intermediate of resorcinol oxidation may conceivably react with manganese, if the demand for manganic ions is heavy, or with itself to form a dimer. In some respects a potentially available surplus of manganic ions would not only be desirable, but perhaps essential for complete oxidation; otherwise, apart from the slow oxidase reaction, the peroxidatic reaction would have to be 100% efficient.

The evidence supporting the general principles of the hypothesis presented in this paper is summarized as follows. Complete decarboxylation and oxidation of 6.67 μM. indoleacetic acid catalyzed by dialyzed wheat leaf extracts, horse-radish peroxidase, or catalase can be attained only when manganese and an appropriate monophenol or resorcinol are present within a critical concentration range. The strict requirement for these cofactors indicates that the Kenten–Mann system involving the peroxidatic oxidation of manganese mediates the oxidation of indoleacetic acid. The fact that only monohydric phenols and resorcinol are active in mediating the oxidation, whereas the other dihydric phenols and pyrogallol are inactive and in contrast inhibit the oxidation by reducing manganic ions, implies a requirement in the oxidation for manganic ions. This is supported by the following: (*a*) Citrate and pyrophosphate, which chelate manganic ions strongly, forming a complex with a low redox potential at pH 6.0, inhibit the reaction completely at a concentration of 10^{-2} *M*. On the other hand, versene, which forms a manganic complex with a relatively high redox potential, has little or no effect on the reaction at this concentration. (*b*) Manganese dioxide decreases significantly the natural lag period shown by dialyzed extracts from summer grown leaves and also the lag period induced by catechol. Moreover, manganese dioxide can partially replace resorcinol as the oxidant of manganese in the presence of a steady supply of hydrogen peroxide produced by an acetaldehyde–aldehyde oxidase system. (*c*) Indoleacetic acid is unstable and undergoes decarboxylation and oxidation at pH 6.0 in the presence of manganic ions produced by the oxidation of manganese by either manganese dioxide or lead dioxide (20). Neither manganese nor manganese dioxide separately have any effect on indoleacetic acid.

The natural lag or induction period has been found to be due to the presence of dialyzable endogenous factors that inhibit the reaction initially by competing for manganic ions, but once oxidized have no further effect. The lag period can be simulated and extended by the presence of as little as 5×10^{-6} *M* catechol, which competes for and is destroyed by manganic ions. Its effect is so delicately balanced that it is almost impossible to duplicate the time period of the lag from one experiment to another. Oxygen and manganese dioxide are the most powerful agents which overcome the catechol induced lag, the former by accelerating the oxidation of resorcinol. Both these agencies

provide a source of manganic ions not involving peroxide derived from indoleacetic acid oxidation. Riboflavin also decreases this lag period significantly, probably by its catalytic effect on the resorcinol 'oxidase' system (21). Light had little or no effect on the catechol-induced lag and no synergistic effect when used in conjunction with riboflavin. However, when the lag period was extended by the addition of natural dialyzable factors, white and blue light, but not red were effective in overcoming this lag period. This effect of light and the effect of oxygen on the naturally induced lag period prompted the present authors earlier (27) to assume the presence in the reaction sequence of a flavoprotein component. As pointed out, the results of recent investigations are not in accord with this concept, although definite proof of its non-existence has not yet been produced.

Another type of competitive inhibition is brought about by such substances as hydroquinone, scopoletin, and riboflavin. These substances do not prolong the lag period but slow down the rate of oxidation below a specified concentration (22). This retardation effect suggests that these substances compete for and reduce manganic ions and their oxidation products are probably capable indirectly of bringing about an oxidation of manganese. Thus the inhibition is dependent on the steady state equilibrium between their oxidized and reduced forms.

A naturally occurring dialyzable factor and maleic hydrazide can substitute for the phenolic compound and mediate the oxidation of indoleacetic acid. Maleic hydrazide in contrast to resorcinol and dichlorophenol does not exhibit a critical optimum concentration at which it is most effective, but its activity increases with increasing concentration (21). It is not known yet whether the concentration of the natural factor is critical since our recent preparations contain impurities that extend the lag period and interfere with the concentration/activity curve. Both maleic hydrazide and the natural factor are presumed to be peroxidase substrates, the oxidized forms of which are capable of oxidizing manganese. The cessation of plant growth brought about by treatment with maleic hydrazide can be explained on the basis of its mediation of the oxidation of indoleacetic acid and therefore the accelerated and persistent destruction of this plant growth hormone.

It is noteworthy that none of the synthetic 'auxins' tested were oxidized by this indoleacetic acid oxidase, although there have been reports of their oxidation by other plant extracts (14, 25). Neither are these substances attacked by manganic ions to the degree that indoleacetic acid reacts (20). Possibly the combination of these two properties contributes to their relative effectiveness as plant growth regulators.

The reaction mechanism as outlined in this paper differs in principle from those postulated by previous investigators (7) in that manganic ions are produced and initiate the reaction sequence. The effects shown by the other systems can be explained on the basis of a direct oxidation of the indoleacetic acid molecule by hydrogen peroxide or in some cases by endogenous manganese

in conjunction with phenolic compounds. The same strict cofactor requirement for the oxidation of indoleacetic acid is shown by horse-radish peroxidase, catalase, and pea epicotyl as well as wheat leaf extracts and it would not be too presumptuous to expect that this same system is ubiquitously employed in the plant kingdom for the inactivation of indoleacetic acid.

The presence of a natural factor in wheat leaves that mediates the oxidation of indoleacetic acid provides strong support for the operation of the Kenten–Mann system *in vivo*. The oxidation of manganese has been shown to occur in the soil (24), in isolated chloroplast systems upon illumination (8, 19), and in the living tissues of *Elodea* (4) and peas (19). The discovery by Kenten and Mann (16) that manganese can be oxidized by peroxidase systems from plants and this demonstration that manganic ions initiate the oxidation of indoleacetic acid links together and establishes a physiological role for the mangano–manganic equilibrium and phenol–peroxidase systems in the growth processes of plants.

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STUDIES ON FUSARIUM WILT OF BANANAS

I. THE BEHAVIOR OF F. OXYSPORUM f. CUBENSE IN DIFFERENT SOILS[1]

By R. H. Stover[2]

Abstract

The behavior in soil of *Fusarium oxysporum* f. *cubense* (E.F.S.) Sny. and Hans., the cause of *Fusarium* wilt or Panama disease of bananas, was studied, using soil dilution plates, soil plates, and buried slide techniques. Soil dilution plates were useful in detecting gross population changes above 500–1000 spores per gram of soil. After six to eight months, initially large populations of *Fusarium* in artificially infested soil had fallen to below 500 spores per gram of soil but could be detected after two years in or around infested glass cloth strips buried in banana plantations. Apparent differences between population counts in loam and sandy loam were attributed to some defect of the soil dilution plate technique.

The soil plate technique consisted in adding 1 gm. of artificially infested soil to a culture dish, moistening the soil with a suitable nutrient, and observing growth and sporulation microscopically or by estimating colony increases on soil dilution plates. An external source of nutrient was essential for germination and sporulation. Among the nitrogen sources tested, 1% solutions of tryptophane and glutamic acid stimulated the abundant production of spores whereas inorganic sources of nitrogen were ineffective. Sporulation was greater in soils dried before plating than in soils maintained at 15–35% saturation. Sporulation was greater in acid sandy loam than in alkaline loam and was abundant below pH 7.0 in all soils. Sporulation in soil plates was sparse or absent below pH 3.5 in acidified loam or above pH 8.8 in sandy loam. The optimum pH for sporulation in acidified loam was about 5.0 and in sandy loam, from about 5.0–7.0. In general, rate of disease spread in soils when they are first planted was correlated with soil texture, pH, and amount of growth and sporulation on soil plates and buried slides. Thus, the soil, rather than the host, appears to be exerting the primary influence on the fungus.

Fusarium grew and sporulated abundantly in decomposing diseased banana pseudostems, on the surface of cut-open rhizomes, and in adhering soil, but did not sporulate freely in sap or in the xylem *in vivo*. Fresh banana sap stimulated the abundant production of spores in soil plates. Evidence is presented suggesting that *F. oxysporum* f. *cubense* also sporulates in nature in saprophytic association with certain soil-plant components.

Introduction

In 1923, Knudson (unpublished reports) noted that the rate of spread of banana wilt caused by *Fusarium oxysporum* f. *cubense* (E.F.S.) Sny. and Hans. was slower in alkaline than in acid soils but heavy liming of infested acid soils did not reduce disease incidence. In 1930, Volk (15) gathered data from 33 banana farms showing the following relationship between soil pH and years of fruit production:

Mean pH of soil	Years of fruit production	Soil designation as related to disease spread
5.6 and below	3–5	"Nonresistant"
5.7–6.2	5–10	"Nonresistant"
6.3–6.5	10–20	"Semiresistant"
6.6 and above	20 plus	"Resistant"

Since a few exceptions were observed, Volk studied soil solutions from these areas and found that, in general, soils with less than 400 p.p.m. total

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soluble salts were favorable to disease spread and those with more than 1000 p.p.m. were unfavorable. Between 400 and 1000 p.p.m., there was no correlation with rate of disease spread. He related the pH, calcium, and sulphate content, and calcium–magnesium ratio with the "resistance," "semiresistance," and "nonresistance" of soils to the spread of banana wilt and concluded that texture influenced but did not control disease spread. In subsequent years, emphasis was placed on the calcium content and calcium/magnesium ratio of soil solutions as the best indicators of rate of disease spread. In general, in soils with high calcium content or calcium/magnesium ratio, disease spread was retarded.

Reinking (7, 8) found that soil texture rather than pH determined disease spread, which was consistently more rapid on light-textured than on heavy-textured soils at pH 5.3–6.0 and was twice as fast on light-textured as on heavy clay soils at pH 7.3–8.0. He invariably found greater numbers of the banana pathogen in light-textured soils (7).

Infestation with banana wilt *Fusarium* had become general in the majority of good banana soils in Central America. These soils are usually alkaline or slightly acid clays, loams, and clay loams. Most of the acid sandy loams were no longer productive by 1930 because of disease. The only known economically feasible method of reclaiming infested soil for growth of the susceptible Gros Michel banana is by flood fallowing and replanting (11, 12) every four to eight years. The reason *Fusarium* and banana wilt spread more rapidly in light-textured, usually acid, low calcium content soils is not known. Studies were recently initiated to determine if the soil influences the pathogen, the host, or both. First detailed results are presented in this paper, a preliminary report of which has already appeared (13).

Materials and Methods

Soils were obtained from the same general areas described previously (10, 11) and from other areas. Samples from the former varied somewhat in physical and chemical characteristics, the range of which is shown in Table I. Dark sandy loam refers to dark brown samples that contrasted with the usual yellow brown of most sandy loam samples. In addition to soil, leaf mold obtained from partially decomposed banana litter on loam

TABLE I

CHARACTERISTICS OF SOILS USED IN EXPERIMENTS WITH *F. oxysporum* f. *cubense*

Soil range	Percentage*			pH range	Source	Wilt disease history
	Clay	Silt	Sand			
Clay – clay loam – loam	18–48	31–46	31–36	7.2–8.4	Mopala Farm, Honduras	Slow, irregular disease spread; in banana production 15–30 years
Loam – sandy loam	6–20	18–33	47–56	5.0–6.6	San Alejo, Honduras	Rapid disease spread; in banana production 3–5 years

**Bouyoucos method of physical analysis; samples were taken to a depth of 6 in.*

soil was also used after it was passed through an 8-mesh-to-the-inch screen. Henceforth, loam refers to Mopala and sandy loam to San Alejo area soils.

As a source of fungus for infesting soils, 10% by volume of corn meal-in-sand cultures of *F. oxysporum* f. *cubense* from two to four weeks old were used. Hereafter, *Fusarium* will refer to this fungus.

Experiments were repeated four to six times with slight modifications in treatment, sequence, or method of recording observations. Materials were maintained at room temperature (74°–92°F.). Growth and sporulation varied from one experiment to another, with certain trends consistent in a series of experiments. Tabular data have been chosen from representative experiments to illustrate these trends. Wherever the identity of isolates of *F. oxysporum* was in doubt, pathogenicity was tested on banana plants growing in 12-in. pots (3, 9).

Three methods used for studying *Fusarium* behavior in soil are briefly described.

Soil Dilution Plate Method

Samples of soil (1 or 10 gm.) were diluted 1:1000, 1:10,000, and 1:100,000 and 1 or 0.5 ml. aliquots transferred to four to six culture dishes which were then poured with peptone-dextrose agar containing rose bengal and streptomycin (10). Counts of fungi were made on the third or fourth day. This method was useful only in detecting pronounced population changes and failed to detect changes in *Fusarium* populations at the relatively low levels (50–100 or less spores/gram of soil) that are most likely to be encountered in nature.

Buried Slide Method

An aqueous suspension of *Fusarium* spores from corn meal-in-sand cultures was added to warm 2% plain agar (pH 5.8). Drops of the agar–spore suspension were placed on slides set upright in glass tumblers filled with screened soil maintained at 15–35% saturation with water. Slides were carefully removed at intervals of two to four weeks, excess soil shaken or tapped off, and agar spots flooded with several drops of a 1% dextrose or amino acid solution in culture dishes. On slides from soils where dextrose stimulated abundant actinomycete growth, glutamic acid was substituted. Growth and sporulation observations under the microscope were begun 18 hr. after the addition of the nutrients. Attempts were made to determine population changes by washing agar drops into soil dilution bottles and making dilution plates. Results were erratic and large numbers of samples were required for significance. Therefore, results were recorded using a scoring system based on relative number and length of hyphae and amount of sporulation observed in 50 microscope fields.

Soil Plate Method

Numerous methods were tried to obtain growth and sporulation records of *Fusarium* in soil *in situ*. A method was devised which produced results

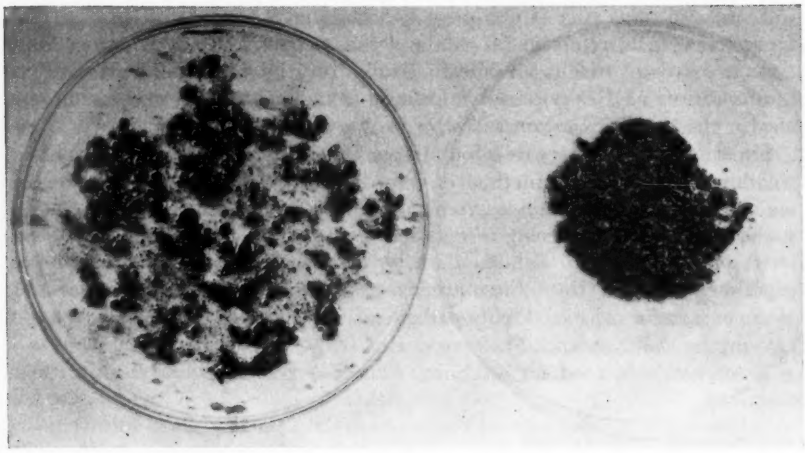


FIG. 1. Soil plate method of studying *Fusarium* behavior in soil. A quantity of artificially infested field soil is placed in the center of the dish and moistened to saturation with a 1% solution of a suitable nutrient such as glutamic acid. The soil on the left was spread out to facilitate observations under the microscope.

considered to approximate most closely *in situ* behavior. Soils under study were infested with a corn meal-in-sand culture of *Fusarium* or occasionally an aqueous suspension of *Fusarium* spores from the same culture but free of sand particles, until 20–30 colonies per ml. of a 1:1000 soil dilution developed on a peptone-dextrose agar plate (10). These soils were then placed in tumblers where moisture content fluctuated from 15–35% saturation. After one to two weeks, and intermittently thereafter, 1 gm. of soil was removed, broken up with a spatula, and spread thinly in a Petri dish (Fig. 1). The soil was moistened to saturation with a nutrient, usually glutamic acid solution containing 1% nitrogen. After 16 and 48 hr., and occasionally after 72 hr. the plates were examined by microscope for the presence of sporulating colonies of *Fusarium*. In addition, soil dilution plates were made and increases in numbers of colonies over check plates moistened with water were recorded. Large amounts of sporulation were reflected in the soil dilution plate colony counts. Nevertheless, abundant sporulation which was not always reflected in increased dilution plate colony counts was frequently observed microscopically. This method, referred to as the soil plate method, differs from Warcup's technique of the same name (16) for isolating soil fungi in that he added 0.005 to 0.0015 gm. of soil to cooled, acidified, Czapek-Dox agar medium.

Results

Changes in Soil Populations Using Soil Dilution Plates

Fusarium populations added to sterile and nonsterile soil in the large quantities required to follow changes with the soil dilution plate technique

always declined. Within six to eight months after addition of 300,000 spores per gram of soil, population had fallen to below 500 per gram. Populations could not be accurately recorded below this level by known techniques. After the first two months, the slope of decline was similar in sterile and nonsterile soils, although the population remained consistently higher in the former (Fig. 2). Populations were always greater in loam than in sandy loam, whether sterile or nonsterile. These soils mixed with 25% by volume of sand had lower populations than control soils free of sand (Fig. 3). There was no apparent reason why populations should be lower in sandy loam than in loam since the former was known to favor disease more than the latter. Physical components of the soil, which could mask differences or similarities in soil populations, or characteristics of the technique, might be responsible for the inconsistent results obtained with soil dilution plates.

In one experiment, four levels of inoculum were used with nonsterile loam and sandy loam and dilution plates made immediately after infestation. Larger populations were apparent in loam than in sandy loam (Table II) when equal or even greater quantities of *Fusarium* were added to sandy loam. The higher populations of other soil fungi in sandy loam may have caused greater competition for space on the soil dilution plate. However, differences between loam and sandy loam were still apparent when sterile soils were used (Fig. 2). The rate of decline of *Fusarium* populations is similar (Fig. 3) and total populations differ owing to unknown factors in the soil dilution plate technique.

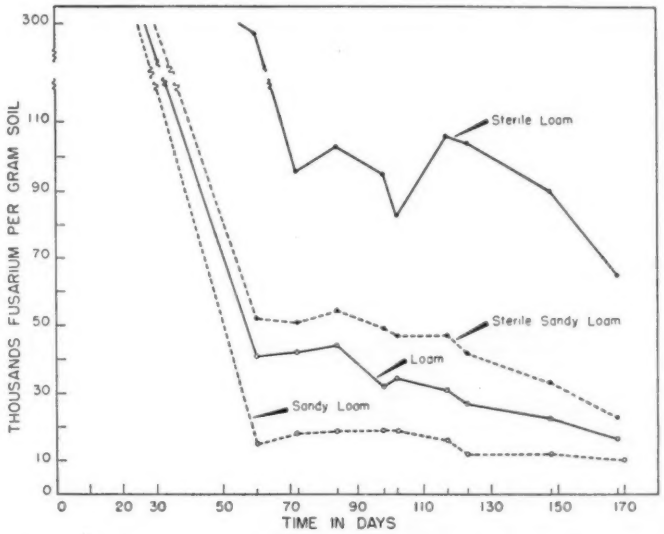


FIG. 2. Population curves, as indicated by soil dilution plates, of *F. oxysporum* f. *cubense* in artificially infested soils maintained in clay pots buried outdoors.

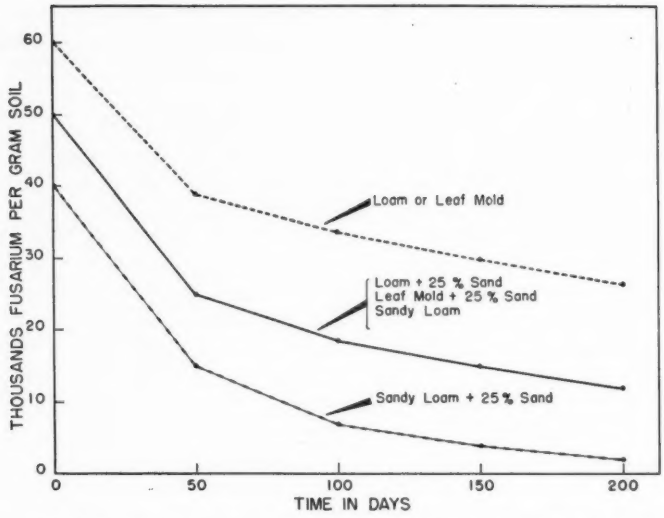


FIG. 3. Populations curves, as indicated by soil dilution plates, of *F. oxysporum* f. *cubense* in artificially infested soils in glass tumblers in the laboratory. Although the same quantities of *Fusarium* were added to all soils, population differences were apparent on soil dilution plates immediately after the fungus was added to the soils.

Sandy loam possesses larger particles than loam which might settle out sooner in soil dilution bottles, carrying *Fusarium* spores with them, and could account for the differences between soils. A 1 gm. sample of soil was diluted 1:100 with water, shaken, and then allowed to settle for two minutes after shaking before being diluted to 1:1000. There was no indication that population differences between soils were due to varied settling rates in soil dilution bottles.

TABLE II

INFLUENCE OF SOIL TYPE, *Fusarium* CONCENTRATION, AND DILUTION ON NUMBERS OF COLONIES OF *F. oxysporum* f. *cubense* ON SOIL DILUTION PLATES FROM SAMPLES TAKEN IMMEDIATELY AFTER INFESTATION OF THE SOIL

Dilution	*Fusarium* concentration*	Colonies per ml. of dilution			
		Loam		Sandy loam	
		Fusarium	Other fungi	Fusarium	Other fungi
1:1000—	1	74	40	6.5	100+
	2	72	26	36	100+
	3	142	29	44	100+
	4	215	36	42	100+
1:10,000	1	2.2	6.0	1.5	74
	2	4.3	7.0	5.5	67
	3	15.0	15.5	2.8	67
	4	14.0	20.0	4.8	98
1:100,000	1	1.0	—	0.2	—
	2	2.4	—	1.0	—
	3	3.2	—	0.4	—
	4	8.2	—	2.0	—

**Grams of corn meal-in-sand culture of Fusarium in 225 cc. of soil.*

Effect of Nutrition on Growth and Sporulation in Soil Plates

Germination and sporulation of *Fusarium* in soil plates did not occur unless an external source of nutrient was added or sterilized soil was used. Infested loam and sandy loam were moistened with a series of 0.1% and 1% aqueous solutions of amino acids, other nitrogen sources, and carbohydrates. Microscopic observations showed that a large number of spores was produced by a relatively small amount of hyphae (Figs. 4–7). Sporulation began within 12 hr. and continued until approximately 40 hr. after the nutrient was added. Hyphal growth and sporulation then ceased and the spores produced did not germinate in the soil plate. Only a small percentage of the viable spores known to be present in the soils, as indicated by soil dilution plate counts, germinated and/or sporulated.

Among the amino nitrogen sources tested, tryptophane and glutamic acid strongly stimulated sporulation (Table III). Urea, ammonium sulphate, and sodium nitrate did not stimulate sporulation. Where sporulation was abundant, it was greater in sandy loam than in loam. Among the carbohydrates, sucrose, glucose, and levulose at 1% concentration were strongly stimulatory in sandy loam but only slightly so in loam. At 0.1% concentration, nitrogen and carbohydrate sources, except occasionally glutamic acid

TABLE III

EFFECT OF NITROGEN AND CARBON SOURCES ON SPORULATION OF *F. oxysporum* f. *cubense* AFTER 48 HR. IN SOIL PLATES

(Average increase of colonies per ml. of a 1:1000 dilution of soil plates over control plates moistened with water)

	Loam	Dark sandy loam
Nitrogen source*		
Tryptophane	23.0	294.8
Glutamic acid	12.2	91.2
Glycine	21.3	51.5
Peptone	10.0	25.0
Leucine	15.7	4.1
Arginine	3.1	5.5
Cysteine	2.1	2.1
Asparagine	3.0	3.3
Cystine	0.1	1.1
Histidine	1.4	1.1
Tyrosine	1.8	0.0
Methionine	1.0	0.0
Lysine	0.4	0.1
Carbon source*		
Sucrose	9	82
Glucose	6	58
Levulose	5	53
d-Sorbitol	1	12
Maltose	4	10
Tryptose	0	5
Lactose	0	0

**Amino acid nitrogen calculated on the basis of nitrogen equivalent to that contained in 1 gm. of asparagine. 1% aqueous solutions of carbon and nitrogen sources were used to moisten soil.*

and tryptophane, rarely stimulated sporulation. Sap exuded from the ends of freshly cut pseudostems stimulated the abundant production of spores.

When six or more samples of soil from different areas within the loam and sandy loam classifications were used, there was a wide variation in amount of sporulation as indicated by soil dilution plates. In general, sporulation was greater in dark brown than in yellow brown sandy loam. In many cases, numerous centers of sporulation were observed microscopically that were not always reflected in increased soil dilution plate colony counts. The reasons for these discrepancies are not known but failure to detect small amounts of sporulation is an inherent weakness in the soil dilution plate method of recording population changes. The average number of sporulating colonies observed microscopically in a series of sandy loam samples was always greater than in a series of loam samples.

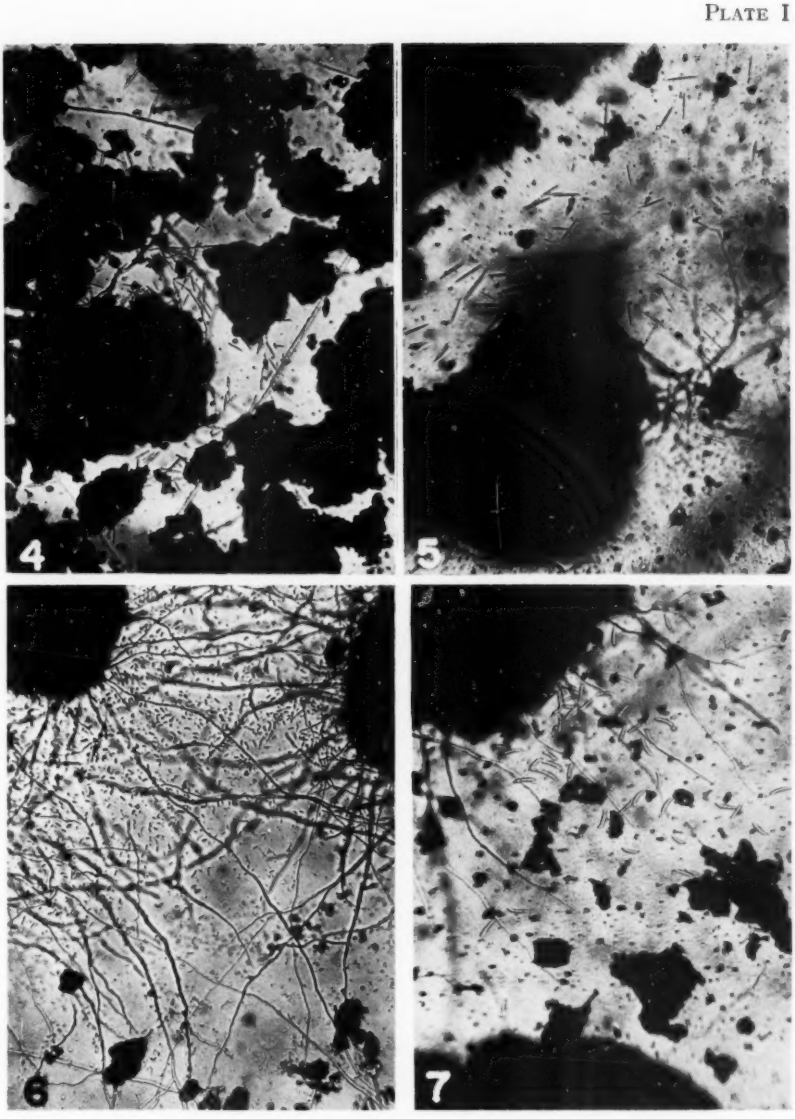
TABLE IV

EFFECT OF pH ON SPORULATION OF *F. oxysporum* f. *cubense* IN SOIL PLATES MOISTENED WITH GLUTAMIC ACID

Soil and additive	pH	Colony increase*
Experiment 1		
Dark sandy loam	5.0†	39
Loam + H_2SO_4	5.0	41
Sandy loam + NaOH	6.0	12.7
Loam + H_2SO_4	7.3	2.7
Sandy loam + $Ca(OH)_2$	7.9	0.7
Sandy loam + NaOH	8.0	2.2
Loam	8.0†	3.0
Sandy loam + $Ca(OH)_2$	8.5	2.5
Experiment 2		
Dark sandy loam	5.1†	44
Loam + H_2SO_4	5.2	39
Sandy loam + NaOH	6.4	83
Loam + H_2SO_4	6.7	74
Sandy loam + $Ca(OH)_2$	7.0	30
Loam + H_2SO_4	7.1	14
Sandy loam + NaOH	7.5	18
Sandy loam + $Ca(OH)_2$	7.6	16
Sandy loam + $Ca(OH)_2$	8.0	2.6
Experiment 3		
Dark sandy loam	5.6†	39
Dark sandy loam + NaOH	6.0	87
Dark sandy loam + $Ca(OH)_2$	6.6	19
Dark sandy loam + NaOH	6.9	100
Dark sandy loam + $Ca(OH)_2$	6.9	35
Loam + H_2SO_4	7.4	0.5
Loam + H_2SO_4	7.6	0.5
Sandy loam + $Ca(OH)_2$	7.9	1.0
Loam	8.0†	4.0

**Increase in number of colonies per ml. of 1:1000 dilution of soil plates moistened with 1% glutamic acid over controls (not shown) moistened with water.*

†Natural pH of field soil samples.



FIGS. 4–7. Sporulation of *Fusarium* in sandy loam soil plates moistened with 1% glutamic acid (250×). FIGS. 4, 5. Macroconidia of *F. oxysporum* f. *cubense*. FIG. 6. Prolific production of microconidia around soil crumbs. FIG. 7. Sporulation of *F. solani* as a "contaminant" in soil plates. These spores are readily distinguishable from the needlelike *F. oxysporum* macrospores and the much smaller microconidia.

Effect of pH on Growth and Sporulation

The pH of dark sandy loam was adjusted upward in series from 5.0 to 9.6 with calcium hydroxide and sodium hydroxide and of loam downward from 8.0 to 2.7 with dilute sulphuric acid. Before using the soils, frequent pH adjustments were required for two to three weeks after which there were only slight changes as a result of soil buffering capacity. Using the soil plate method and glutamic acid as a source of nutrient, sporulation was 5–20 times greater in soils below pH 7.0 than above pH 7.0 (Table IV). It was not possible to distinguish consistent differences in the range 5.0–7.0 or 7.0–8.5 because of the wide variations in soil dilution plate colony counts. In a subsequent series of experiments, fresh banana sap was used as a source of nutrient (Table V). Sporulation was sparse or absent below pH 3.6 and above pH 8.8. In highly buffered, acidified loam, optimum pH for sporulation was about 5.0; in less highly buffered sandy loam, optimum pH ranged from 5.0–7.0 although occasionally sporulation was abundant at pH 7.4–7.8.

TABLE V

EFFECT OF pH ON SPORULATION OF *F. oxysporum* f. *cubense* IN SOIL PLATES MOISTENED WITH FRESH BANANA SAP

(Colonies per ml. of 1:1000 dilution of soil plated)

Adjusted with:	pH*	Banana sap	Water	Increase†
		Loam		
H_2SO_4	2.7—3.2	0	0	0
	3.6—3.8	11.2	4	7.2
	5.2—6.2	136.2	4.8	131.4
	6.1—6.6	56.8	5.6	62.4
	6.7	56.0	7.1	52.9
	6.9	106.7	7.4	99.3
Unadjusted	7.2—7.3	69.7	1.3	68.4
		Sandy loam		
Unadjusted	5.0—5.5	75.0	4.1	70.9
$Ca(OH)_2$	6.0	89.5	4.6	84.9
	6.5—7.0	45.8	3.3	42.5
	7.3—7.4	63.6	3.0	60.6
	7.8—8.1	17.3	3.4	13.9
	8.1—8.5	20.8	4.4	16.4
NaOH	6.2—6.6	98.4	6.8	91.6
	7.0—7.4	108.7	9.6	99.1
	7.5—7.8	73.5	7.6	65.9
	8.0—8.1	58.3	11.1	47.2
	8.7—9.0	30.8	16.0	14.8
	9.3—9.6	13.0	9.7	3.3

**Range of pH of replicates at beginning and end of experiment.*
†Average increase in number of colonies from soil plates moistened with fresh banana sap over controls moistened with water.

Using acidified loam and buried slides, the lower the pH down to 4.0, the greater was the amount of growth and sporulation. In most instances, sporulation did not occur on slides previously maintained at pH 7.4–8.0 and moistened with 1% dextrose solution (pH 5.8). A small increase in sporulation often occurred on the same slides moistened with 1% glutamic acid solution (pH 3.5), which lowered the initial pH (5.8) of the agar drops slightly. When the soil pH was lowered to 6.7, a pronounced increase in sporulation occurred on slides with dextrose and glutamic acid.

Growth and Sporulation in Different Soils

In light-textured soils with a history of rapid disease spread, *Fusarium* grew and sporulated more abundantly than in alkaline heavy-textured soils with a history of slow disease spread when first planted (Table VI). There was no correlation between rate of disease spread and amount of sporulation in a heavy-textured alkaline soil that had been flood fallowed to eradicate a *Fusarium* infestation.

To determine the extent of sporulation in the physical fractions of loam and sandy loam soils, air-dry samples were put through Tyler soil physical analysis screens of 200, 160, 100, and 65 meshes per inch. The fractions were then infested with *Fusarium*, placed in jars, brought to approximately 25% saturation with water, and incubated two weeks before soil plates were made. The loam fractions did not favor sporulation, except the coarsest tested. Sporulation occurred in all sandy loam fractions.

Soils in mature banana plantations are covered with a 1 to 3 in. layer of leaf mold derived from decayed banana trash. This material did not favor sporulation of *Fusarium* in soil plates (Table VI) and was studied in more detail using buried slides. *Fusarium* grew on slides previously held in leaf

TABLE VI

RELATION OF TEXTURE, pH, PREVIOUS WILT HISTORY, AND AMOUNT OF SPORULATION IN SOIL PLATES AFTER ADDITION OF 1% DEXTROSE

% Composition*			Soil type	pH	Rate of wilt spread†	Sporulation	
Sand	Silt	Clay				14 hr.	42 hr.
47	33	20	Loam	5.7–6.0	Rapid	Fairly abundant	Abundant
28	38	34	Clay	6.0	Rapid	Fairly abundant	Very abundant
23	42	35	Clay	6.2	Rapid	Fairly abundant	Very abundant
32	45	23	Clay loam	6.0	Rapid	Abundant	Abundant
36	47	17	Loam	7.8	Rapid	Abundant	Abundant
21	31	48	Clay	8.3	Slow	None	Trace
32	37	31	Clay	7.6	Slow	None	Trace
20	39	41	Clay	7.7	(*a*) Slow‡	Trace	Trace
					(*b*) Rapid	Trace	Trace
—	—	—	Leaf mold	8.0	—	None	None

**Mechanical analysis—Bouyoucos method.*

†*Rate at which spread when soils first planted: soils in banana production for 5 years or less had a rapid rate of disease spread; soils in production for 10 years or more had a slow rate of disease spread.*

‡(*a*) *When soils first planted;* (*b*) *after flood fallowing.*

mold at moisture levels up to 50% saturation but it very infrequently or never sporulated. At 75% saturation and above, however, the fungus did not survive three weeks in leaf mold. Banana leaf mold apparently is not a favorable environment for *Fusarium* growth and sporulation.

Experiments to determine if the sporulation differences observed microscopically on soil plates could be detected by colony counts in soil dilution plates showed a wide variation between experiments and among soil samples. On the average, sporulation was greater in sandy loam than in loam as indicated by soil dilution plates although the difference was not as great as that observed microscopically (Table VII). Sporulation was greater in soil that had dried out for three to six days before testing than in soil maintained at 25% saturation. The relation between concentration of *Fusarium* in soil and amount of multiplication on soil plates was also studied (Table VIII). Doubling the concentration of the fungus in soil did not result in consistent increases in colony counts.

TABLE VII

VARIATION IN SPORULATION AMONG DIFFERENT SAMPLES OF SANDY LOAM AND LOAM IN SOIL PLATES

(Average colonies per ml. of 1:1000 dilution of soil plated)

Soil type	Soil previously dry			Soil previously 15–25% saturated		
	Glutamic acid	Water	Increase*	Glutamic acid	Water	Increase*
Sandy loam	10.0±2.1	0.3±.17	9.75±2.2	5.8±1.4	0.8±.18	5.1±1.3
Loam	3.0±1.3	1.0±.35	2.3 ±1.3	3.8± .77	2.8±.79	1.2± .48

**Average increase in number of Fusarium colonies on soil dilution plates from soil plates moistened with 1% glutamic acid over those moistened with water.*

TABLE VIII

INFLUENCE OF *Fusarium* CONCENTRATION AND SOIL MOISTURE ON SUBSEQUENT MULTIPLICATION OF *F. oxysporum* f. *cubense* IN SOIL PLATES

(Colonies per ml. of 1:1000 dilution of soil plated)

Fusarium concentration*	Loam moistened with:			Sandy loam moistened with:		
	Glutamic acid	Water	Increase	Glutamic acid	Water	Increase
			Soil previously dry			
1	2.7	2.5	0.2	5.2	1.0	4.2
2	9.0	3.2	5.8	7.6	2.0	5.6
3	8.5	3.7	4.8	28.2	1.2	27.0
4	19.0	8.0	11.0	38.7	4.1	34.6
			Soil previously 15–25% saturated			
1	1.7	10.1	0	4.9	1.0	3.9
2	4.0	8.5	0	5.2	2.2	3.0
3	5.7	3.0	2.7	10.2	2.8	7.4
4	10.2	11.2	0	7.1	1.9	5.2

**Grams of corn meal-in-sand culture of Fusarium per 225 cc. of soil.*

The effect of soil sterilization on *Fusarium* multiplication in loam and sandy loam is shown in Table IX. Percentage increases in populations were greatest in nonsterile sandy loam (3536%), intermediate in sterile loam (571%) and sandy loam (620%), and least in nonsterile loam (177%). The addition of 0.25 and 0.50 gm. of zinc sulphate or manganese sulphate per 250 cc. of soil did not influence the amount of multiplication.

In buried slide studies (Table X), the pH of agar drops on the slide was taken with the "one-drop" Beckman pH electrode. In drops moistened with distilled water, the pH varied from 5.5 to 6.7 in sandy loam and from 7.8 to 8.0 in loam. In drops moistened with 1% glutamic acid solution, the pH fluctuated from 4.3 to 6.4 in sandy loam and from 7.7 to 8.3 in loam. Growth and sporulation were greater on slides from sandy loam (Table X, Figs. 8 and 9). In experiments not shown in Table X, sporulation differences between loam and sandy loam were more marked where 1% dextrose was used as a nutrient.

TABLE IX

MULTIPLICATION OF *F. oxysporum* f. *cubense* ON STERILE AND NONSTERILE SOIL PLATES

(Colonies per ml. of 1:1000 dilution after 48 hr.)

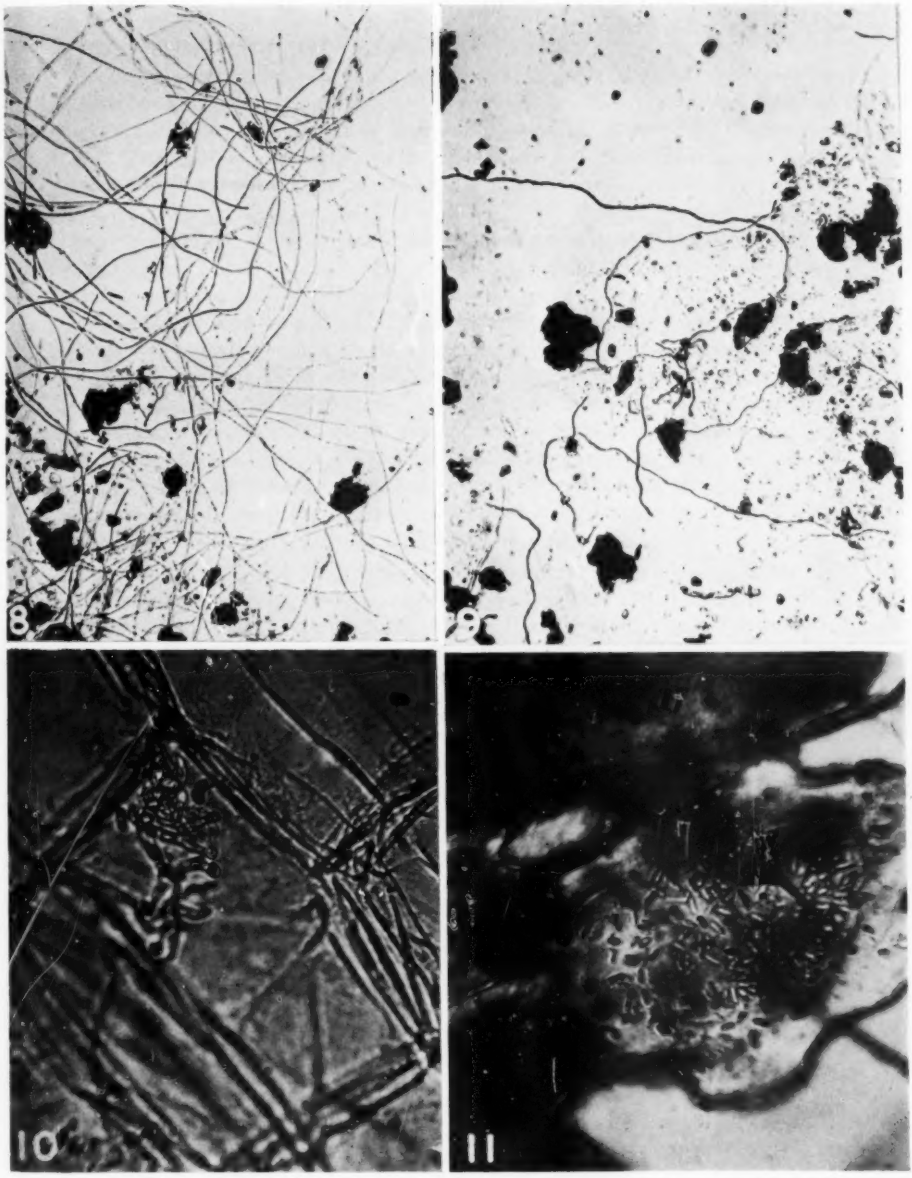
Sterile loam moistened with:			Nonsterile loam moistened with:		
Glutamic acid	Water	Increase	Glumatic acid	Water	Increase
75.0	11.2	64.0	2.5	0.9	1.6
9.0	2.4	7.6	0.9	0.02	0.8
Sterile sandy loam moistened with:			**Nonsterile sandy loam moistened with:**		
Glutamic acid	Water	Increase	Glutamic acid	Water	Increase
209	29	180	40	1.1	38.9
40.2	6.2	35	4.2	0.09	4.2

TABLE X

RELATIVE GROWTH AND SPORULATION OF *F. oxysporum* f. *cubense* ON SLIDES REMOVED FROM DIFFERENT SOILS AFTER 41 DAYS

Loam samples				Sandy loam samples								
1	2	3	4	1	2	3	4	5	6	7	8	9
Sporulation*												
3	9	0	5	21	25	22	28	19	16	21	28	23
Hyphal growth*												
17	18	5	14	27	29	19	26	30	21	27	26	28

**Sporulation and hyphal growth scored from 0 to 10 for minimum and maximum observed 48 hr. after slides were moistened with 1% glutamic acid. Scores are total of three experiments.*



FIGS. 8, 9. Growth of *F. oxysporum* f. *cubense* at the periphery of infested agar drops (250×) on slides placed in soil for three weeks, removed, and moistened with 1% dextrose. Note dense hyphal growth from periphery of agar drop formerly in sandy loam (Fig. 8) contrasted with that formerly in loam (Fig. 9).

FIGS. 10, 11. Sporulation of *F. oxysporum* f. *cubense* in cells of decomposing diseased banana tissue (450×). FIG. 10. Microconidia being produced on end of short conidiophores. FIG. 11. Dense mass of microconidia often found within a cell.

Source and Survival of Inoculum in Soil

Previous studies showed that only a small percentage of the roots of diseased banana plants contained *F. oxysporum* f. *cubense*, even in advanced stages of disease (14). A high inoculum potential, in addition to various soil factors (7), is necessary for rapid disease spread. Therefore, studies were made of possible sources of inoculum and survival of the inoculum in soil.

Nondecomposed diseased pseudostems (average height, 15–20 ft., average diameter, 8–12 in.) had a sparse growth of fine hyphae in the discolored vascular strands, and sporulation did not take place although an occasional microconidium was found. The copious amounts of sap exuded from the cut ends of diseased pseudostems were free of conidia although hyphal fragments were present. *Fusarium* colonies infrequently developed on agar plates to which the sap was transferred. In the field, the freshly cut surfaces and exuded sap were colonized by *F. roseum* and sometimes by *F. solani* and *F. moniliforme* but not by *F. oxysporum* f. *cubense*. Vascular fibers containing hyphal strands of *Fusarium* adhered to knife blades which had cut diseased pseudostems. There was no evidence from field experiments that such blades were an effective means of distributing the fungus.

Fusarium in decomposing diseased pseudostem material grew into the cortical tissue adjoining the vascular strands and sporulated, usually within individual cells (Figs. 10 and 11). In later stages of tissue breakdown, *Fusarium* grew and sporulated on the walls of the hollow cavities formed within pseudostem sheaths. Besides *F. oxysporum* f. *cubense*, *F. solani*, *Cephalosporium* sp., and in some cases *F. roseum* were also found. Apparently hyphae of *F. oxysporum* f. *cubense* are confined to the vascular strands until pseudostem tissue begins to break down. Then they grow through this decaying tissue and produce large quantities of spores.

In infected roots removed from soil, *Fusarium* did not grow throughout the stele or into the cortex. The cortical tissue decayed rapidly and was colonized primarily by an unidentified oospore-forming phycomycete, *Rhizoctonia* sp., *Cephalosporium* sp., and *F. solani*. Apparently, production of spores by *F. oxysporum* f. *cubense* in decaying roots is not abundant.

The large, hard rhizome (average diameter, 9–13 in.) of diseased banana plants decays slowly and is heavily parasitized by *Fusarium*. About three-quarters of the rhizome is stelar with a high concentration of xylem tissue. Diseased rhizomes were cut open and covered with a 1/4 in. layer of moist field soil. Within 24 hr., *F. oxysporum* f. *cubense* grew through this layer and abundantly sporulated on the soil surface. As the rhizome tissue decayed from the exposed surfaces inward, *Fusarium* was found occasionally sporulating in individual cells surrounding the vascular strands near the cut surface but not in the firm undecomposed tissue in the center of the rhizome.

Fusarium sporulated abundantly in decaying banana pseudostems and probably in rhizomes but not in sap *in vivo* as reported by Rishbeth (9). Most of the xylem elements in these tissues are invaded by the fungus; as

decay advances, the pseudostem and rhizome could be colonized by *Fusarium*. A 10 mm. square portion of colonized pseudostem has yielded 100,000–7,000,000 colonies when macerated and cultured on agar dilution plates. Tremendous quantities of spores are presumably released into the soil from decaying diseased tissue. Most of the spores probably perish in the natural decline of large *Fusarium* populations added to soil. A small percentage must survive to be carried to and infect adjacent living banana roots.

Populations below 500 spores per gram of soil cannot be accurately determined by soil dilution plates and the low concentration of spores in soil makes detection on plates remote. Therefore, strips of glass cloth from 2% corn meal-in-sand cultures of *Fusarium* were buried in the surface 2 inches of soil in banana farms. The glass cloth disintegrated slowly and solved the problem of diluting out in soil space as far as choosing soil samples likely to contain *Fusarium* was concerned. The glass cloth strips were removed periodically and *Fusarium* population estimated by plating the strips and the adhering soil separately. *Fusarium* was consistently found in buried strips and in adhering soil after nine months. After two years, the glass cloth strips were partially disintegrated and usually only occasional colonies of *F. oxysporum* f. *cubense* were still present. Infrequently, however, a glass cloth strip was recovered with a *Fusarium* population almost as great as that present one to three months after burial. Studies are continuing to determine if multiplication had occurred saprophytically.

Discussion

The soil dilution plate technique is too crude and has too many inherent defects to measure *Fusarium* population changes that probably occur in soil microhabitats. This technique can detect only gross changes in survival and, in some cases (12), growth rates at population levels far in excess of those in nature. In the present studies, soil dilution plate colony counts fluctuated widely. Some samples from areas with a rapid rate of disease spread supported 5 to 10 times more sporulation than those from areas of slow disease spread. Other samples from the same area showed little or no increase in sporulation over samples from areas of slow disease spread. Nevertheless, the average amount of sporulation in 10 or more samples from the former area was two to four times greater than from the latter. These variations among soil samples may be due to differences in characteristics influencing fungus growth as well as to the soil dilution plate technique. Sporulation was often detected microscopically in soil plates but was not reflected in increased colony counts on dilution plates. The soil dilution plate technique did not consistently detect *Fusarium* in or around diseased plants (14), an observation confirmed by Rishbeth (9).

It is difficult to rationalize the present results on populations in different soils with those of Reinking (7) unless his isolates refer to the section Elegans (*F. oxysporum*) and not specifically to *F. oxysporum* form *cubense*. Hansford

(3) and Rishbeth (9) in pathogenicity experiments with isolates of *F. oxysporum* from bananas indicate that Reinking (7) was apparently dealing with this entire group rather than specifically with *F. oxysporum* f. *cubense*. If such is the case, then the studies now reported, which show greater sporulation of *F. oxysporum* f. *cubense* in sandy loam, are probably applicable to the entire Elegans section in the soils studied.

Fusarium populations in soil gradually decline and would become extinct unless multiplication occurred with or without a host. Initially high populations in decomposing diseased bananas may provide a few survivors after two years in the absence of interim multiplication. However, it is inconceivable that *Fusarium* known to survive 20 years in soils free of bananas could do so unless multiplication occurred on wild hosts or saprophytically in soil. Park (6) has shown that fungus spores from a wide range of common soil fungi, including the saprophyte *F. roseum*, decompose when placed in soil. A population was maintained in soil only by addition of various organic soil substrates. The decline of large populations of *Fusarium* added to field soil plus the improbability that scattered single spores can cause the rapid spread of disease that often occurs when the host is re-established (12) indicate that sporulation may occur in the absence of pathogenesis.

The soil plate technique described in this paper shows that nutrition is one of the important determining factors in *Fusarium* sporulation in soil. Apparently adequate nutrition can overcome an inhibitory or fungitoxic effect present in nonsterile soils. Although Hessayon (4) found no fungitoxins in aqueous soil extracts, some inhibitory factor preventing *Fusarium* germination in nonsterile soil is overcome by soil sterilization and reduced by an adequate nutritional stimulus. Dobbs and Hinson (2) reported that drying soils reduced a widespread soil fungistasis. Sporulation in soil plates was increased in soils dried before plating.

Not only nutrition but also pH and soil type strongly influence *Fusarium* growth and sporulation. Most light-textured banana soils are acid and the heavy-textured soils are usually alkaline, pointing to a correlation of pH and calcium content with soil texture. Reinking (8), however, correlated soil texture with rate of disease spread in different soils of the same pH. In Central America, disease spreads rapidly in some alkaline light-textured soils and either rapidly or slowly in some acid heavy-textured soils, invalidating the consistent correlation of soil texture and pH with rate of disease spread in the field. This evidence suggests that other factors as yet unknown are also involved. *Fusarium* grows and sporulates well in alkaline (pH 7.8) light-textured loam with a history of rapid disease spread. Also, pH studies in soil plates indicate that the optimum pH can occur over a wider range in sandy loam than in loam.

In natural soils sporulation could result from a nutritional stimulus, i.e., the liberation of amino acids into the rhizosphere (5), provided by the host or other plants. These plants or other agents overcome spore dormancy enforced by soil fungitoxins and/or inadequate nutrition. If dormancy

were broken and conditions were not propitious for sporulation or infection, the fungus would perish (1). In certain heavy-textured soils studied, even though germination occurred, growth and sporulation were inhibited because of a high pH and other undetermined factors. The broad correlation between rate of disease spread in virgin soils and amount of growth and sporulation of *Fusarium* indicated that the soils studied and not the host were exerting the primary influence on the fungus.

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COMPARATIVE STUDIES OF PHOTOSYNTHESIS IN NOSTOC MUSCORUM AND CHLORELLA PYRENOIDOSA[1]

By K. A. Clendenning, T. E. Brown, and H. C. Eyster

Abstract

Methods are described for the continuous culturing of *Nostoc muscorum* and for the manometric measurement of its photosynthesis. *N. muscorum* required a more dilute and more alkaline medium for growth and for measurement of its photosynthesis than *Chlorella pyrenoidosa*. Although lacking organized chloroplasts, *N. muscorum* exhibited higher photosynthetic capacity at 25°C. in terms of cell numbers and chlorophyll content than *C. pyrenoidosa*. The photosynthetic capacities of *N. muscorum* and *C. pyrenoidosa* were not significantly different on a solids basis. Saturating light intensities, optimum and maximum temperatures, activation energies, induction periods, and inhibition by phenylurethane were similar for photosynthesis in *Nostoc* and *Chlorella*. Aerobic induction was not abolished by an atmosphere of pure oxygen, or by glucose feeding.

Photosynthesis in *Nostoc* was abolished by exposing the filaments to weakly acidic media, and usually was injured by washing them with neutral or mildly alkaline potassium-free media. The lyotropic series for the prevention of washing injury in *Nostoc* was $K > Rb > NH_4$, Li, Na. This washing effect is discussed in relation to the chloride effect on the Hill reaction, the magnesium effect on *Azotobacter* respiration, as well as the effect of potassium on the bioelectric properties of *Nitella*, to which it appears related.

Introduction

Photosynthesis in the primitively organized blue-green algae has considerable interest from a comparative viewpoint, but its kinetics has received little attention (50). When our studies were begun four years ago, the photosynthetic capacities of blue-green algae were quantitatively unknown, and the conditions for their measurement had not been defined. Quantitative data meanwhile have been provided on the photosynthetic capacities of *Anabaena* and *Anacystis* under different conditions by Kratz and Myers (41, 44), who have also provided some data on *Nostoc*. Emerson and Lewis (27) previously had established that light absorbed by phycocyanin in *Chroococcus* is used as effectively in photosynthesis as light absorbed by chlorophyll. Duysens (21) showed that phycocyanin transfers absorbed light energy with *ca.* 100% efficiency to chlorophyll *a*. Frenkel, Gaffron, and Battley (32) effected hydrogen adaptation in *Chroococcus* and *Synechococcus;* Thomas and DeRover (53) showed that phycocyanin is an integral part of the water photolysis apparatus in *Synechococcus;* Calvin and Lynch (11) and Thomas (52) provided electron photographs of its grana. Early products of $C^{14}O_2$ assimilation in four species of blue-green algae were shown to be the same as in *Chlorella* by Norris, Norris, and Calvin (45). Brown, Webster, and Frenkel (7, 57) investigated influences of light and other factors on respiration in *Anabaena*. Assimilation of atmospheric nitrogen in *Nostoc*, *Anabaena*, and other blue-green algae has been studied extensively (e.g. 1, 31).

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Preliminary to our investigations of photosynthesis in *Nostoc muscorum*, its mineral requirements were defined by Eyster (28–30). The assimilation of C^{14} labelled carbon dioxide, formate, acetate, urea, and other organic compounds in *N. muscorum* was investigated at the Southern Research Institute, Birmingham, Ala., as one part of the Kettering Foundation program (2, 3). Investigations of *N. muscorum* at Yellow Springs, Ohio, have included research on its mineral metabolism (28–30), its chloroplastin, phycocyanin, and phycocyanobilin (12, 18, 38), comparative studies of photosynthesis and the Hill reaction in healthy and potassium-deficient *Nostoc* (14), and the comparative studies of photosynthesis in *Nostoc* and *Chlorella* with which this and other reports are concerned (8, 12, 13).

Materials and Methods

Algae

Nostoc muscorum, Gerloff strain, was obtained from Dr. G. C. Gerloff, University of Wisconsin; *Chlorella pyrenoidosa*, Emerson strain, was obtained from Dr. R. Emerson, University of Illinois, and from Dr. Jack Myers, University of Texas. Slant cultures were maintained on 2% agar in Emerson–Lewis solution (26) plus 0.5% glucose and 0.2% peptone. The mineral nutrient solution developed by one of us (H.C.E.) for *Nostoc* was prepared with reagent chemicals and deionized distilled water as follows: solution I, 5.19 gm. $NaHCO_3$, 0.24 gm. K_2HPO_4 in 4 liters; solution II, 1.00 gm. $MgSO_4 \cdot 7H_2O$, 1.38 gm. $Ca(NO_3)_2 \cdot 4H_2O$, plus 8 ml. trace element solution in 4 liters; solution III, 1.00 gm. $FeSO_4 \cdot 7H_2O$ in 1 liter, prepared freshly each week; trace element solution, 126 mgm. $Na_2MoO_4 \cdot 2H_2O$, 20 mgm. $CoCl_2 \cdot 6H_2O$, 19 mgm. $Cu(NO_3)_2 \cdot 3H_2O$, 22 mgm. $ZnSO_4 \cdot 7H_2O$, 285 mgm. H_3BO_3, and 180 mgm. $MnCl_2 \cdot 4H_2O$ in 500 ml. The complete medium for *Nostoc* consisted of 500 ml. solution I, 500 ml. solution II, and 7 ml. of solution III, and had a pH of 7.0–7.5 under the conditions of the continuous cultures. The liquid media for *C. pyrenoidosa* were those of Emerson and Lewis (26) and of Warburg (55), which had pH values of *ca.* 6.0 and 4.0, respectively, in the culture vessels. The algae were grown in 1 liter pure cultures on a shaker apparatus similar to that of Benson *et al.* (5) (25° ± 1°C., bubbled with 5% CO_2 in air (35–50 ml./min.)). Illumination (1300 ft-c.) was provided by 40 w. white fluorescent lamps placed immediately above and below the cultures. The algae were harvested according to the method of Benson *et al.* (5). Cell numbers were determined with a haemocytometer to ±5% (20 slides/harvest). Packed cell volume (p.c.v.) was determined in duplicate by centrifuging previously concentrated suspensions to minimum volume in calibrated hematocrit tubes (30 min., 2600 R.C.F.).

After acclimatization, the *Chlorella* populations doubled every six hours, and *Nostoc* doubled every seven or eight hours on a daily cycle. Growth of *Chlorella* was as rapid as in any previous work, and that of *Nostoc* was much higher than in most earlier studies. The filamentous *Nostoc* suspensions were more difficult to sample accurately than *Chlorella:* after thorough

mixing, *Nostoc* suspensions were kept in motion while aliquots were withdrawn with rapid pipettes. Sedimentation of *Nostoc* filaments was more rapid in the centrifuge than was the sedimentation of *Chlorella*, and *Nostoc* pellets obtained in p.c.v. measurements were less compact, and more spongy, swelling noticeably within 10 min. For that reason, p.c.v. was read immediately after centrifuging. The gelatinous *Nostoc* filaments have a greater creeping tendency than *Chlorella* cells, in shaking culture flasks and in Warburg vessels. *Nostoc* is retained quantitatively on coarse filter paper which passes *Chlorella* cells. Static *Nostoc* suspensions settle in darkness, but the filaments rise to the surface when illuminated, presumably a result of entrapped oxygen bubbles.

One milliliter of packed *N. muscorum* filaments contained an average of 1.25×10^{10} cells (extreme range, 1.0–1.8×10^{10}). Diameters of the vegetative *Nostoc* cells were 4–5 μ. Slightly larger unpigmented heterocysts accounted for *ca.* 3.5% of the *Nostoc* cells. There were 10–80 cells (median *ca.* 30) per uniseriate *Nostoc* filament. One milliliter of packed *C. pyrenoidosa* cells from acclimatized daily harvests typically contained 5.1×10^{10} cells, diameters 2–5 μ. The proportion of small *Chlorella* cells was high, the cell diameters averaging 3 μ. Solids per ml. p.c. in *Nostoc* (120–140 mgm.) were only 50% of those observed in *Chlorella* (220–280 mgm.). Chlorophyll *a* is the only chlorophyll detectable in *Nostoc* by chromatography on sucrose (18); the chlorophyll content of *Nostoc* in terms of p.c.v. was about one-fifth that of *Chlorella* (1.9–2.3 mgm. chlorophyll *a* per ml. packed *Nostoc* vs. 10–15 mgm. chlorophyll ($a + b$) per ml. p.c. of *Chlorella*). On a solids basis, *Nostoc* contains 1.5–1.9% chlorophyll, as compared to 4.6–6.8% in *Chlorella*. *c*-Phycocyanin is the only bilichromoprotein detectable in *Nostoc* by tricalcium phosphate gel chromatography (18). Employing a spectrophotometric method calibrated against crystalline phycocyanin from *Nostoc*, the *c*-phycocyanin content of *Nostoc* solids was found to be 8.7% (18). The tetrapyrrole content of *c*-phycocyanin is at least 4% (18), so the phycocyanobilin content of *Nostoc* on a solids basis was at least 0.35%. Carotenoids are abundant in *Nostoc* but these were not measured or identified.

Manometry

Photosynthesis and respiration was measured by the direct and two-vessel methods described below, as well as by external diethanolamine buffering, on which our findings and recommended conditions will be reported subsequently. The incandescent light sources were closely spaced 300-w. projector flood lamps placed four inches below the horizontal window of a large shelf-type thermostat. Infrared was removed with two $\frac{1}{4}$ in. layers of heat-absorbing glass which were cooled with an air blast. Calibrated neutral filters were placed above the infrared filters. Light intensities at the bottoms of the Warburg vessels were measured with an Eppley thermopile and G.E. foot-candle meter. Temperature control was within $\pm$ 0.01°C. at 25°C. and within 0.02°C. at other temperatures.

The bicarbonate buffer selected experimentally for *Chlorella* was *M*/10 No. 9 prepared with 85 parts *M*/10 $NaHCO_3$ and 15 parts *M*/10 K_2CO_3; this buffer was diluted to 0.025 *M* for direct (single vessel) measurements of photosynthesis in *Nostoc*. Two- and multiple-vessel studies of blue-green algae had not been conducted previously. *Nostoc* and other blue-green algae require neutral or mildly alkaline media; part of the carbon dioxide always is chemically bound, and the proportion of bound CO_2 must remain constant for accurate two-vessel manometry. Equilibrium bicarbonate concentrations for a range of pH values and CO_2 levels were calculated with the equation, $\log (HCO_3^-) = pH - pK^1 + \log \alpha\ (2.17 \times 10^{-3})$ (54). The optimum pH for growth and photosynthesis in *Nostoc* was found to be 7.0–8.0; under 5% CO_2 in air at 20°C., the required bicarbonate molarities for this pH range are 7.73×10^{-3} to 7.73×10^{-2} *M*. At the adopted intermediate pH of 7.5, the required bicarbonate concentration is 0.0244 *M* under 5% CO_2. For two- and multiple-vessel applications, *Nostoc* macronutrient solution was adjusted to pH 7.5 and its bicarbonate content was raised to 0.025 *M* prior to equilibration under 5% CO_2 in air. Similar conditions were selected independently by Kratz and Myers (41) for *Anabaena* and *Anacystis*. For corresponding measurements on *Chlorella* the cells (5–10 $mm.^3$) were suspended in 5 ml. of their culture solution, minus iron and trace elements, and were equilibrated under 5% CO_2 in air. Photosynthetic capacities by the two-vessel method were based on rates of carbon dioxide and oxygen exchange observed over 40–60 min. periods of continuous, saturating illumination, after 10 min. in light. Cell densities were kept low to eliminate mutual shading-vessel geometry effects (5–10 $mm.^3$ cells/rectangular vessel). The vessels (constructed by E. Machlett and Son, New York) had unequal volumes and were provided with gassing vents. Accuracy of the two-vessel measurements was assessed by four-vessel combinations involving widely different gas/liquid ratios (agreement within 5%, one-hour measurements). The photosynthetic capacities of the cells in each vessel were shown to be the same by subsequent measurements in No. 9 buffer, using the same cells, liquid volumes, and vessels (agreement within 1%, one-hour measurements). The routine centrifuging treatments were shown to have no effect on the photosynthetic capacities of *Nostoc* and *Chlorella* (13). Assimilation of molecular nitrogen by *Nostoc* did not occur under the conditions of our photosynthesis measurements; its potential magnitude is discussed below.

Results

Light Saturation

The white (incandescent) light intensity which saturated photosynthesis in very dilute *Nostoc* and *Chlorella* suspensions at 25°C. was *ca.* 1600 ft-c., 17,300 lux, or 7.5×10^4 ergs/$cm.^2$/sec. (Fig. 1). These data refer to cells in 0.025 *M* and 0.1 *M* No. 9 buffers, respectively, but the saturating light intensity was equally high for cells suspended in their nutrient solution under 5% CO_2. Similar saturating light requirements for photosynthesis were

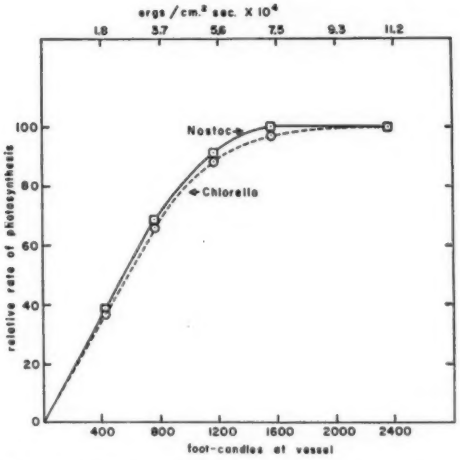

FIG. 1. Light intensity curves for photosynthesis in *Nostoc muscorum* and *Chlorella pyrenoidosa*.

observed previously on *Chlorella ellipsoidea* (51) and on *Ulva lactuca* fronds (17) at 25°C., and on *Chlorella vulgaris* at 23°C. (19); at least 16,000 lux is required to saturate growth in *Anabaena* at 25°C. (1). Light saturation of photosynthesis by much lower intensities was observed by Kratz and Myers (41) on *C. pyrenoidosa*, *Anabaena*, *Anacystis*, and *Nostoc* at 25°C.; the organisms used in the latter study were grown at the same temperature but were grown at a lower light intensity than we employed.

Temperature Relations

Measurements of photosynthetic capacity at each temperature (0°–45°C.) were made on fresh aliquots withdrawn successively from refrigerated stock cultures. The algae were stored overnight at 0°C. before rate measurements in No. 9 buffers were begun, and they were in the thermostat for a total of 30 min. before being illuminated at each temperature. Each series of determinations at 12 temperatures was completed within 20–24 hr. Thermal inactivation in the range 35°–40°C. occurs gradually, the apparent temperature optimum being influenced to some extent by the length of the reaction period. Photosynthesis in *Nostoc* was found to remain approximately constant for at least 40 min. beyond induction at 35°C., however, the "optimum" temperature being the same for the first and second halves of 40-min. reaction periods. Twenty-five minute illuminations accordingly were adopted, rate of photosynthesis at each temperature being calculated from oxygen evolution after five minutes in light. The rate data for *Nostoc* and *Chlorella* were made to coincide at 25°C. (Fig. 2).

Photosynthesis in *Nostoc* and *Chlorella* was rapidly inactivated at 42.5–45°C., and in this respect there was no appreciable difference between these algae

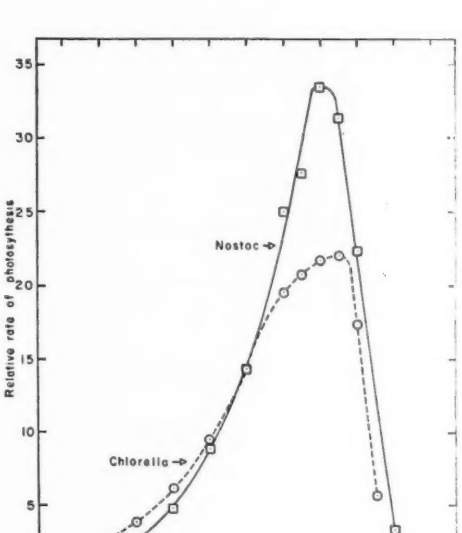

FIG. 2. Temperature relations of photosynthesis in *Nostoc muscorum* and *Chlorella pyrenoidosa*.

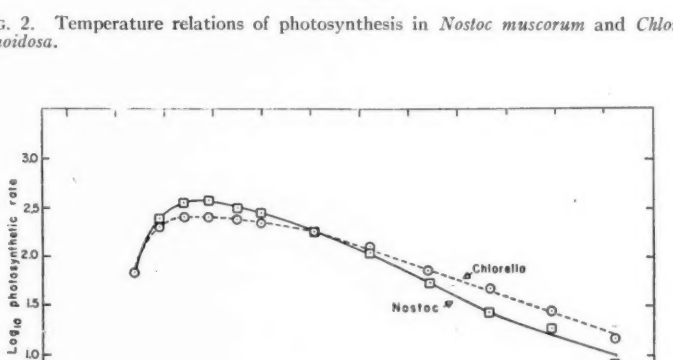

FIG. 3. The logarithm of the rate of photosynthesis plotted against $1/T$ absolute for *Nostoc muscorum* and *Chlorella pyrenoidosa*.

when grown as described (Fig. 2). Respiration in both organisms was less sensitive to high temperatures than photosynthesis, and had a broader temperature optimum. The optimum temperature for photosynthesis in *Nostoc* and *Chlorella* was 35°C.–37°C. (*ca.* blood temperature), or similar to that observed previously for photosynthesis in *C. vulgaris* (19) and in *Scenesdesmus* (47).

Logarithms of the photosynthetic rates are plotted against reciprocals of the absolute temperatures in Fig. 3. The data fall almost on straight lines between 0° and 30°C. for *Nostoc*, and between 0° and 25°C. for *C. pyrenoidosa*, the curves crossing at 25°C. *Nostoc* showed lower relative rates than *Chlorella* below 25°C., and higher relative rates in the range 30°–40°C. *Chlorella* therefore is favored by the use of 25°C. or lower temperatures vs. 30°–35°C. in comparisons of its photosynthetic capacity with that of *Nostoc* and presumably of other blue-green algae. The different slopes of the *Nostoc* and *Chlorella* data between 0°–25°C. are indicative of differences in activation energy (Fig. 3). Employing the equation $E=(RT_2 \cdot T_1)/(T_2-T_1)$ ln k_2/k_1 the calculated activation energy for *Nostoc* photosynthesis over the range 0°–25°C. is 16,730 cal./mole as compared to 16,000 cal./mole for *Chlorella*. These values are temperature characteristics of the limiting dark reaction which controls the photosynthetic rate at saturating light intensities. Corresponding data for *C. vulgaris* provided by Emerson (22) yielded Arrhenius curves which were distinctly concave downward, μ increasing from 16,000 to 31,000 as the temperature was decreased from 26° to 4°C. Nearly linear Arrhenius curves were obtained for photosynthesis in *C. vulgaris* between 10°–30°C. by Craig and Trelease (19), who also found that the activation energy for photosynthesis is the same in D_2O and H_2O. With the exception of Emerson's data below 10°C. for *C. vulgaris*, activation energies for photosynthesis in the range 0°–30°C. have usually fallen in the range 15–20,000 (19, 22).

Temperature coefficients (Q_{10}) for photosynthesis and respiration in *Chlorella*, *Nostoc*, and other organisms rise above 3.0 as the temperature is lowered toward 0°C., a general property of enzymatic processes. High Q_{10} values at low temperatures apparently have little basic significance, their cause lying in this method of expressing temperature relations. If the temperature were reduced until the reaction stops, Q_{10} for the first 10° interval above this temperature becomes ∞; as the "denominator" reaction rate approaches zero, Q_{10} rises toward infinity. The temperature coefficient for photosynthesis, respiration, and other enzymatic processes falls at high temperatures (> 20°C.) as a result of thermal inactivation.

pH Relations

Chlorella pyrenoidosa is commonly grown at pH 4.0 (55), and its photosynthetic efficiency and capacity usually are measured under mildly acidic conditions. Under a constant and saturating supply of CO_2, photosynthesis in *C. pyrenoidosa* was found by Emerson, Green, and Webb (25) to be unaffected

by pH in the range 4.6–8.9. The insensitivity of photosynthesis in *Scenedesmus* to pH changes was established by Ouellet and Benson (48); CO_2 assimilation was not eliminated entirely by lowering the pH to 1.6 or by raising it to 11.4, and upon restoring a pH of 7.0 in the same suspensions, the full rate of photosynthesis was rapidly recovered (48).

The main difficulty of establishing the pH relations of photosynthesis directly for any organism is the accompanying changes in ionic strength, carbonate, bicarbonate, and CO_2 concentration in the medium; bicarbonate drops to zero as the pH is reduced below 7.0, and the carbon dioxide supply drops to zero as the pH is raised above 10.0. Kratz and Myers (41) measured photosynthesis of *Anabaena* and *Anacystis* in a series of bicarbonate–carbonate buffers, the rate dropping sharply below pH 7.0 and above pH 10.0. We obtained similar data for *Nostoc muscorum* by the same method, but as Kratz and Myers pointed out, the meaning of such data is uncertain. Direct demonstration of pH effects upon *Nostoc* photosynthesis under otherwise constant conditions became feasible when it was found that these effects are essentially irreversible. For the experiments reported in Fig. 4, 10^{-3} *M* KCl in "deionized" water was adjusted to different pH values with NaOH and HCl. Potassium was included in these "incubation" fluids to avoid the washing injury to which *Nostoc* photosynthesis otherwise is subject, and which is discussed below. *Nostoc* filaments were washed twice and resuspended in each solution; after one hour, the filaments were transferred to 0.025 *M* No. 9 buffer and their photosynthetic capacity was determined at 25°C. Photosynthesis in *Nostoc* was reduced 60% following exposure to a pH of 5.5 (unadjusted distilled water containing 10^{-3} *M* KCl), and was eliminated by prior exposure to a pH of 4.0. Incubating the cells for one hour in 10^{-3} *M* KCl was without effect in the pH range 7.0–8.0, and had only a small inactivating effect up to pH 11.0. Upon raising the pH of the wash liquid from 11.0 to 11.5, photosynthesis by *Nostoc* in 0.025 *M* No. 9 buffer was abolished. Photosynthesis

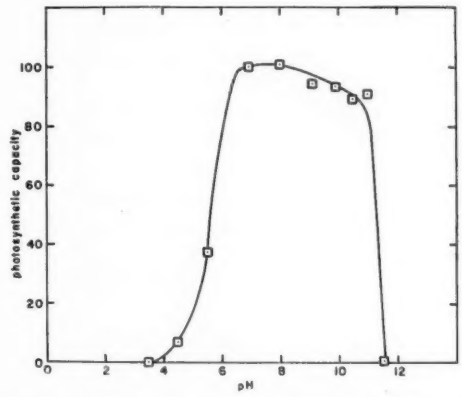

FIG. 4. The pH stability relations of photosynthesis in *Nostoc muscorum*.

in *Nostoc* thus is irreversibly injured by exposure to mildly acidic (pH <7.0) and to strongly alkaline media (pH >11.0), the cellular buffering mechanism presumably failing under these conditions.

Washing Injury

Photosynthesis in *Nostoc* is injured by the slight acidity of ordinary distilled water (pH 5.5), but upon neutralization of the distilled water (pH 7.0–8.0) with $NaHCO_3$, the water commonly causes a second type of injury which results from the absence of potassium. In preparing *Nostoc* and other blue-green algae for tagging experiments, nutrient salts have been removed by washing the cells with distilled water (2, 3, 45); this preparatory treatment has little effect on *Chlorella* but is very apt to damage *Nostoc* for the foregoing reasons.

Injury of *Nostoc* photosynthesis by washing with neutral and alkaline potassium-free media was discovered while we were seeking methods for research on potassium-deficient *Nostoc* cultures (14). When either freshly harvested or stored *Nostoc* filaments grown on complete medium were washed one or more times on the centrifuge with neutralized potassium-free distilled water, or with 0.01–0.1 *M* sodium bicarbonate – carbonate buffers, their photosynthetic capacity in 0.025 *M* No. 9 buffer was reduced, and could be eliminated by extending the washing treatment. *Nostoc* was always subjected to a continuous washing with complete nutrient throughout its growth, and the harvested cells were not injured by washing on the centrifuge in complete nutrient or in (85 Na : 15 K) 0.025 *M* No. 9 buffer. This washing injury is irreversible or only slowly reversible, being expressed to a similar extent when the washed *Nostoc* filaments are subsequently suspended either in Na–K or "all-sodium" bicarbonate buffers for measurements of photosynthesis. It does not involve visible pigment changes, and has little effect on the Hill reaction capacity. Washing injury by potassium-free media is not exhibited to any comparable extent by *Chlorella*.

Potassium was shown to be the only mineral element involved in this phenomenon by the following process of elimination, the washing media being adjusted to pH 7.0–8.0 to avoid the H^+ effect already discussed; photosynthesis was measured in 0.025 *M* No. 9 buffer prepared with sodium salts. Single 10 ml. washes of 10 $mm.^3$ cells were employed in most of these experiments, the injury increasing with more extensive washing. The washing injury developed when *Nostoc* was extracted with the normal strength of its trace elements plus iron, but not with the major nutrient salts (complete nutrient less trace elements). The element which prevented washing injury therefore was a constituent of the major element solution (sodium bicarbonate, potassium monohydrogen phosphate, magnesium sulphate, and calcium nitrate). This solution could be diluted fourfold before washing injury became detectable (8). When the major nutrient salts were tested one at a time, all at the same ionic strength and pH, washing injury was prevented only by potassium monohydrogen phosphate, the sole source of potassium

in *Nostoc* growth medium. Upon comparison of sodium phosphate, potassium phosphate, and potassium chloride, all at the same ionic strength and pH, washing injury developed with the sodium salt but not with either of the potassium salts (8). Finally, provision of 5–15 p.p.m. potassium in neutralized distilled water abolished the injurious effect of washing as effectively as complete nutrient solution; when the potassium is supplied as $2 \times 10^{-4}M$ $KHCO_3$, the two injurious effects of distilled water on *Nostoc* are both eliminated.

The potassium concentration which eliminates washing injury is reported in Fig. 5. (Deionized distilled water was adjusted to pH 7.0 with $NaHCO_3$; potassium chloride was dissolved in aliquots of this solution to provide the desired concentration range.) At potassium concentrations below 5 p.p.m., extensive washing reduced the photosynthetic capacity of *Nostoc* in 0.025 *M* No. 9 buffer, 85 Na: 15 K, and its photosynthetic activity was abolished when potassium was completely withheld in these washing experiments (Fig. 5). The potassium concentration required to prevent washing injury in resting *Nostoc* cells was the same as had been indicated in the dilution series on complete nutrient; it was also similar to the potassium requirement for *Nostoc* growth (14).

Nostoc is more sensitive to high potassium chloride concentrations than *Chlorella*, its photosynthetic capacity falling as the potassium chloride concentration was raised from 10^{-2} *M* to 10^{-1} *M*. The optimum range of potassium concentrations for *Nostoc* media are *ca.* 2×10^{-4} *M* to $2 \times 10^{-3}M$.

The specificity of potassium in preventing this washing injury was demonstrated by comparing the effects of $3.5 \times 10^{-4}M$ potassium, rubidium, ammonium, lithium, and sodium chlorides as washing fluids, in parallel with complete nutrient solution. The chloride ion had previously been shown to be without effect on this phenomenon. The basal medium was deionized distilled water containing $10^{-4}M$ $NaHCO_3$, pH 7.0, to which the foregoing salts were added. Potassium was the only monovalent cation found

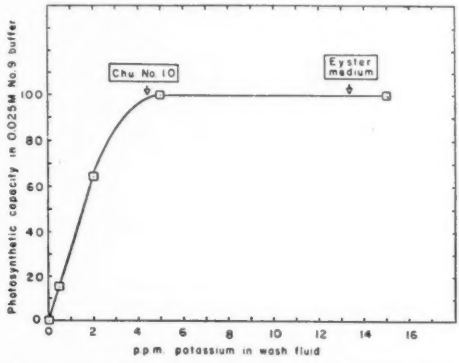

FIG. 5. Potassium concentration requirement for the prevention of washing injury in *Nostoc muscorum*.

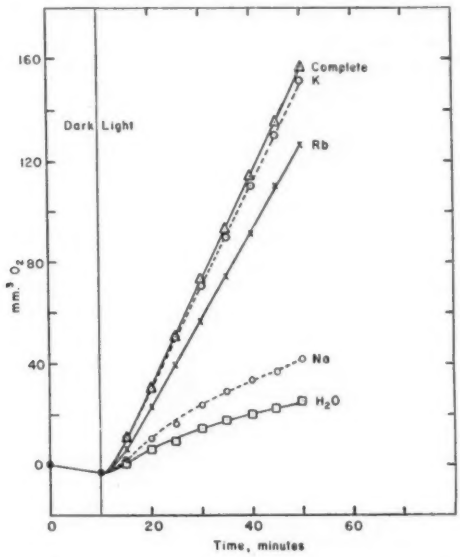

FIG. 6. Relative effectiveness of potassium, rubidium, and sodium in preventing washing injury in *Nostoc muscorum*.

equivalent to complete nutrient solution; rubidium ranked second, being significantly less effective than potassium (Fig. 6). The remaining cations were less effective than rubidium, but their individual ranking remains undecided. The lyotropic series for the prevention of washing injury to *Nostoc* photosynthesis is K > Rb > Na, Li, NH_4.

Effects of washing were compared on photosynthesis in *Chlorella* and *Nostoc*, and upon the quinone–Hill reaction in *Nostoc* (Table I). The photosynthetic capacity of *Chlorella* was reduced only 30% by seven thorough washes; under the same conditions, photosynthesis in *Nostoc* was reduced 60% by two

TABLE I

EFFECT OF WASHING *C. pyrenoidosa* AND *N. muscorum* WITH WATER UPON PHOTOSYNTHETIC AND HILL REACTION ACTIVITY. 5 MM.³ CELLS, 10 ML. WASHES, PHOTOSYNTHESIS MEASURED IN 0.1 *M* AND 0.025 *M* NO. 9 BUFFERS, 85 NA : 15 K. HILL REACTION MEASURED IN 3 ML. 0.05 *M* PHOSPHATE, pH 7.0, CONTAINING 1 MGM. *p*-BENZOQUINONE

No. of washes	*Chlorella* photosynthesis	*Nostoc* photosynthesis	*Nostoc* Hill reaction
0	100	100	100
1	82	91	—
2	—	37	—
3	80	0	93 (with 0.01 *M* KCl) 77 (without 0.01 *M* KCl)
7	68	0	—

washes and was completely eliminated by three washes, without perceptible changes in pigmentation. (No visible loss of pigments or discoloration is associated with washing injuries to *Nostoc* caused either by potassium deprivation or by mild acidity.) The Hill reaction capacity remained high in *Nostoc* cells whose photosynthetic capacity had been eliminated by washing (Table I); a related phenomenon occurs when potassium deficiency is induced during growth of *Nostoc* (14). The small reduction in Hill reaction activity that attended washing was reversed upon incubation of the washed *Nostoc* cells in *M*/100 KCl, which caused no recovery of photosynthetic capacity. If the washing injury had been caused by phycocyanin migration, photosynthesis and the Hill reaction would have been eliminated simultaneously. Potassium does not participate directly in water photolysis by isolated chloroplasts (15, 16, 33); those parts of the complete photosynthetic apparatus in *Nostoc* which are not directly involved in water photolysis are presumably the site of the washing injury induced by potassium-free media.

Sensitivity to potassium-deficient wash fluids was strikingly apparent in most of the *Nostoc* populations examined over a period of four years; there were several instances, however, in which photosynthesis in *Nostoc* was insensitive to washing. The factors responsible for the occasional resistance of *Nostoc* to washing injury have not been identified. Metabolic control of potassium traffic in living tissues is in turn dependent upon oxygen supply (9); although heavy centrifuging itself has little effect on the photosynthetic capacity of *Nostoc* or *Chlorella* (13), development of oxygen deficiency in the centrifuge offers a possible basis for interactions between potassium-deficient media and centrifuging treatments. The washing injury of *Nostoc* photosynthesis resembles Osterhout's "potassium-effect" on the bioelectric properties of *Nitella* (46). The relative susceptibility of different blue-green algae to injury by potassium-free media remains unknown; the insensitivity of *Anabaena* and *Anacystis* to washing with Na–K bicarbonate buffers noted by Kratz and Myers (41) is shared by *Nostoc*, which is readily injured by potassium-free bicarbonate buffers. This phenomenon probably is related to the beneficial effects of potassium in No. 9 buffers; its further study should assist in the gaining of a full understanding of the role of potassium at the cellular level.

Bicarbonate Buffers for Nostoc

In selecting the best bicarbonate buffer for *Nostoc*, effects of buffer molarity, bicarbonate/carbonate and Na/K ratios, and effects of culture medium concentration upon bicarbonate buffer molarity requirements were investigated. *Nostoc* was subjected to dilution series of No. 9, No. 11, and pure bicarbonate buffers (Table II); photosynthesis in *Nostoc* was doubled when the molarity of these three buffers was reduced from 0.1 *M* to 0.025 *M*, and remained approximately constant in the range 0.025–0.01 *M*. (*Chlorella* photosynthesis by contrast was reduced 15–20% upon substitution of 0.025 *M* for the usual 0.1 *M* No. 9 buffer.)

TABLE II

PHOTOSYNTHESIS BY *Nostoc muscorum* IN SODIUM BICARBONATE BUFFERS OF DIFFERENT MOLARITIES PREPARED IN *Nostoc* MINERAL NUTRIENT SOLUTION (MM.^{3} O_2/HOUR/10 MM.^{3} *Nostoc* CELLS AT 25° C., 800 FT-C.)

CO_2, molarity	$NaHCO_3$		No. 11 buffer 95 HCO_3^-:5 $CO_3^=$		No. 9 buffer 85 HCO_3^-:15 $CO_3^=$	
	Rate	pH	Rate	pH	Rate	pH
0.018			30.0	9.0	29.8	9.2
0.033	24.6	8.75	29.4	9.0	28.5	9.2
0.058	22.7		20.1	8.9	13.5	9.2
0.108	12.0	8.8	10.7	8.8	13.4	9.1

Photosynthesis in *Nostoc* was increased when sodium bicarbonate buffers were prepared in *Nostoc* culture medium, but not when the buffers were prepared with mixtures of sodium and potassium salts. The observed rates of photosynthesis were slightly higher in 0.025 *M* No. 9 than in 0.025 *M* No. 11 buffer; upon variation of the Na:K ratio in 0.025 *M* No. 9 buffer, higher rates were observed with 85 Na: 15 K than with 15 Na: 85 K or with pure sodium or potassium salts. The selected medium therefore was the No. 9 buffer (85 Na: 15 K) employed by Emerson and Lewis on *Chlorella* (26), diluted to one-fourth strength. This medium is as satisfactory for *Nostoc* as the 0.1 *M* buffer is for *Chlorella*. Possible errors in measurements of oxygen evolution arising from inadequate CO_2 buffering in these media (39) were found negligible when the gas/liquid ratios were varied fivefold. Both organisms show higher rates of photosynthesis in nutrient solution under 5% CO_2 than in these carefully selected bicarbonate buffers. Photosynthetic capacities of *Nostoc* in 0.025 *M* No. 9 buffer were stable during one hour measurements, and usually were in the range 15–22 ml. O_2/ml. p.c./hour, Q_{O_2}, 110–160 at 25°C. The corresponding rates of photosynthesis in *M*/10 No. 9 buffer were *ca.* 50% lower, in agreement with Kratz and Myers' data for *Nostoc* in the latter medium. *Oscillatoria* freshly collected from a local lake showed a photosynthetic Q_{O_2} value of 85 at 30°C. in 0.025 *M* No. 9 buffer during the first half hour, which dropped 50% during the next 30 min.; *Oscillatoria* apparently is even more sensitive to bicarbonate than *Nostoc*.

Warburg's growth medium for *Chlorella* has about the same molarity as his *M*/10 No. 9 buffer. Mineral nutrient solutions used in culturing blue-green algae by contrast are very dilute. Preliminary experiments indicated that tolerance of 0.1 *M* No. 9 buffer by *Nostoc* can be increased by adapting the cells to concentrated salt solutions during their growth (Fig. 7). The concentration of *Nostoc* growth medium was varied from 0.003 to 0.1 *M*, through uniform changes in concentration of the major elements (Fig. 7). After three days in the most concentrated growth medium, the observed rates of photosynthesis in 0.025 *M* and 0.1 *M* No. 9 buffer became the same (Fig. 7). The three day growth rate was reduced 20% by the highest salt

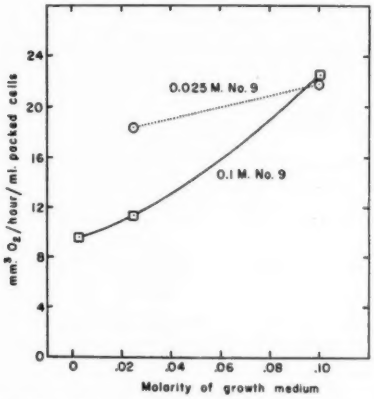

FIG. 7. Photosynthesis by *Nostoc muscorum* in 0.025 *M* and 0.1 *M* No. 9 buffer in relation to molarity of growth medium.

concentration, and the average cell size was increased 10%; 0.025 *M* No. 9 buffer however is superior to 0.1 *M* for *Nostoc* cultured in the dilute nutrient solutions in which it grows rapidly.

Bicarbonate Buffers for Chlorella

Numerous bicarbonate buffers have been used in studies of *Chlorella* photosynthesis. Warburg's original *M*/10 No. 9 buffer was prepared with sodium salts; Emerson and Arnold (23) used potassium salts, and Emerson and Lewis (26) employed a mixture of sodium and potassium salts (85 Na: 15 K). Myers (42) used *M*/10 No. 11 buffer prepared with potassium salts. Pratt (49) compared numerous *M*/10 bicarbonate buffers on *Chlorella* that had been washed three times with distilled water. The rate of photosynthesis rose for *ca.* 10 hours in potassium bicarbonate/carbonate, fell in sodium bicarbonate/carbonate, and showed intermediate responses to intermediate Na/K ratios. Warburg *et al.* (56) recently reported that *M*/5 No. 11 buffer (95 parts $NaHCO_3$, 5 parts Na_2CO_3) plus traces of $MgSO_4$ and $Ca(NO_3)_2$ under an atmosphere of 2% CO_2 in air is superior to all other bicarbonate buffers for direct measurements of quantum efficiency in *Chlorella*. From their results, photosynthesis should proceed twice as rapidly at limiting light intensities in *M*/5 No. 11 vs. *M*/10 No. 9, and should proceed as rapidly at saturating light intensities in *M*/5 No. 11 as in nutrient under 5% CO_2.

All of the foregoing buffers, with additional variations, were compared on *C. pyrenoidosa* from our continuous cultures. For one or two hour measurements in saturating light at 25°C., none was superior to the *M*/10 No. 9 buffer (85 Na: 15 K) employed by Emerson and Lewis (26). Observed photosynthetic capacities of *Chlorella* cultured in Warburg's medium (55) in our experience were never higher in *M*/5 No. 11 buffer (56) than in the Emerson–Lewis buffer, the data obtained with the "new" buffer (56) being corrected for incomplete CO_2-buffering.

Myers (43) showed that the concentration of *Chlorella's* major nutrient salts can be varied 10-fold without altering the growth rate or photosynthetic capacity in nutrient solution. (Changes in salt concentration were made gradually in the Myers–Clark apparatus.) Myers' study (43) did not include effects of sudden changes, as when *Chlorella* is transferred from mineral nutrient solutions of different strengths to *M*/10 No. 9 buffer. The observed sensitivity of *Nostoc* to this buffer, and its apparent dependence upon the salt concentration of the growth medium prompted comparisons of *Chlorella* grown in Warburg's medium (55) and in the more dilute Emerson–Lewis medium (26). After acclimatization, equally high growth rates and photosynthetic capacities (60 ml. O_2/ml. p.c./hour at 25°C.) were attained by the Emerson strain grown in these two media (Table IV). The effect of No. 9 buffer on photosynthesis in *Chlorella* grown in either medium was similar to that observed on *Nostoc*, the observed photosynthetic rates being about 50% higher in nutrient under 5% CO_2 than in No. 9 buffer (Tables III, IV). This difference persisted when the temperature was reduced from 25°C. to 10°C., and when the light intensity was reduced stepwise to 10% of saturation. These comparisons included successive measurements on the same cells in the same vessels, first in nutrient under 5% CO_2, and subsequently in No. 9 buffer. Equal rates of photosynthesis in nutrient under 5% CO_2 and in

TABLE III

PHOTOSYNTHESIS AND RESPIRATION OF *Nostoc muscorum*, GERLOFF STRAIN, AT 25° C. IN NUTRIENT SOLUTION UNDER 5% CO_2 AND IN 0.025 *M* No. 9 BUFFER (ML. GAS EXCHANGE/ML. P.C./HOUR, TWO-HOUR MEASUREMENTS)

	Indirect method		$\frac{CO_2}{O_2}$	Direct method, oxygen
	Oxygen	Carbon dioxide		
	(dark respiration)			(dark respiration)
No. 289, one-day cycle, 1.9 mm.³ cells/ml. harvest, 100 mm.³ cells/vessel	0.56	0.535	0.96	0.53
	(net photosynthesis)			(net photosynthesis)
No. 288, one-day cycle, 2.3 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	32.6	32.8	1.01	20.8
No. 290, one-day cycle, 2.0 mm³. cells/ml. harvest, 5 mm.³ cells/vessel	30.7	30.3	0.99	—
No. 291, one-day cycle, 1.9 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	36.3	34.0	0.94	—
No. 311, three-day cycle, 5.7 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	14.4	14.2	0.99	12.2
No. 313, first one-day cycle, 2.9 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	V_f = 5 ml. 21.0 V_f = 3 ml. 20.4	21.8 20.8	1.04 1.02	13.2

TABLE IV

PHOTOSYNTHETIC CAPACITIES OF *C. pyrenoidosa*, EMERSON STRAIN, AT 25° C. IN NUTRIENT SOLUTION UNDER 5% CO_2 AND IN NO. 9 BUFFER (ML. GAS EXCHANGE/ML. P.C./HOUR, ONE HOUR MEASUREMENTS)

Sample	Indirect method		$\frac{CO_2}{O_2}$	Direct method, oxygen
	Oxygen	Carbon dioxide		
Emerson strain, grown in Emerson–Lewis medium				
No. 362, one-day cycle, 1.6 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	61.1	57.8	0.945	41.6
No. 361, two-day cycle, 3.4 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	56.7	53.3	0.94	41.2
No. 182, three-day cycle, 2.8 mm.³ cells/ml. harvest stored overnight at 0° C., 10 mm.³ cells/vessel	47.6	44.9	0.94	30.5
No. 343, three-day cycle, 3.7 mm.³ cells/ml. harvest, 10 mm.³ cells/vessel	39.8	40.7	1.02	21.2
Emerson strain, grown in Warburg medium				
No. 360, two-day cycle, 2.6 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	65.3	61.5	0.94	47.8
No. 363, one-day cycle, 1.5 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	56.7	53.3	0.94	41.2
No. 307, one-day cycle, 1.75 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	49.7	46.7	0.94	32.1
No. 314, three-day cycle, 2.6 mm.³ cells/ml. harvest, 5 mm.³ cells/vessel	V_f = 5 ml. 42.0 V_f = 3 ml. 42.6	40.7 39.4	0.97 0.93	29.1

No. 9 buffer were observed only on *Chlorella* cells of low activity from very dense suspensions (6 mm.³/ml.), harvested after four days' growth. Effects of nutrient salt concentration (Emerson–Lewis vs. Warburg growth medium) upon photosynthesis in No. 9 buffer were not apparent in *Chlorella* harvests of equivalent photosynthetic capacities (Table IV). These comparisons refer to acclimatized *Chlorella* suspensions containing a high proportion of small cells. Tamiya *et al.* (51) demonstrated that changes in the proportionate numbers of large and small *Chlorella* cells are associated with large changes in photosynthetic capacity and other properties.

Photosynthesis in 0.1 M KCl

Brackett *et al.* (6) measured the quantum efficiency of *Chlorella* photosynthesis in 0.1 *M* KCl, which inhibits photosynthesis in *Nostoc*. According to

Greenfield (35), *Chlorella* photosynthesis is inhibited slightly by exposure to 0.1 *M* KCl, the degree of inhibition rising with further increases in KCl concentration. Photosynthesis in *Chlorella* from the present cultures was not inhibited significantly (> 5%) at either limiting or saturating light intensities when 0.1 *M* KCl was substituted for nutrient solution under 5% CO_2.

Photosynthetic Induction

Initial time courses of photosynthesis in *Nostoc* and *Chlorella* were determined in 0.025 *M* and 0.1 *M* No. 9 buffers, using Warburg's original method for the study of induction, which involves intermittent illumination. Similar time courses were observed on these algae by this method, as well as with an adaptation of the carbonic anhydrase boat-method (vessel shaken at high speed while attached with pressure tubing to a stationary manometer). The induction period was magnified by anaerobic dark pretreatments in *Nostoc* and *Chlorella*. The aerobic induction periods for both algae at 25°C. were of less than two minutes' duration, and were not eliminated by a pure oxygen atmosphere. Brackett *et al.* (6) reported that induction losses are reduced when *Chlorella* is maintained in 1% glucose. We have incubated *Chlorella* aerobically in 1–4% glucose for 1–24 hr. in darkness and subsequently measured initial time courses of photosynthesis in No. 9 buffer plus glucose, and in some cases plus *M*/100 phosphate. Bacteria-free conditions were maintained throughout. Dark respiration was increased as much as 500% by sugar-feeding, but the aerobic induction period was not altered significantly. Similar findings have been reported recently by Damaschke *et al.* (20).

Inhibition by Phenylurethane

The surface-active urethanes cause a reversible inhibition of *Chlorella* photosynthesis at limiting and saturating light intensities (50). Degree of inhibition by urethanes varies with their molecular size, *ca.* 3×10^{-4} *M* phenylurethane effecting 50% inhibition of photosynthesis in *Chlorella* (50) as well as in the marine red alga *Gigartina* (24). Photosynthesis in *Nostoc muscorum* shows the same response to phenylurethane (Fig. 8). The similar concentration requirement for 50% inhibition by phenylurethane in green and red algae as well as in chloroplast-free blue-green algae suggests a common mode of action. Presumably the urethanes penetrate the protein–lipid interphases within chloroplasts and grana, becoming associated with the chlorophyll films (38).

Nitrogen Assimilation Capacity

Atmospheric nitrogen assimilation by *Nostoc* and other blue-green algae presumably is powered by the same forms of biochemical energy that are utilized in nitrate assimilation and carbon dioxide assimilation (reduced pyridine nucleotides and high-energy phosphates provided either by respiration or by photochemical means). N_2-fixation introduces a potential source of error in manometric studies of photosynthesis. It was eliminated in the

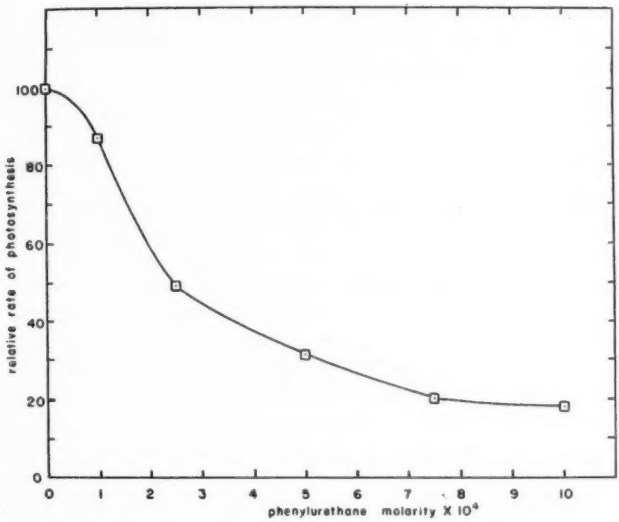

FIG. 8. Inhibition of photosynthesis in *Nostoc muscorum* by phenylurethane.

present work by supplying nitrate in the growth and test media, but a quantitative estimate of its potential magnitude was needed for reference purposes. *Nostoc* grown in Eyster's medium contains *ca.* 50% carbon and 8% nitrogen, and under 1300 ft-c. white light at 25° C. it doubles every seven to eight hours. One milliliter of packed cells of *Nostoc* grown in this way contains *ca.* 130 mgm. solids; a 100% increase in cell solids during the generation period necessitates the assimilation of 65 mgm. carbon and of 10.4 mgm. nitrogen by an inoculum of 130 mgm. solids, or by an average of 195 mgm. solids during the doubling period (1.5 ml. p.c.). Assimilation of 65 mgm. carbon is equivalent to 121 ml. CO_2, and 10.4 mgm. nitrogen equals 8.4 ml. N_2. The average assimilation rates during the growth period expressed as Q_{CO_2} and Q_{N_2} (mm.^{3} gas/hr./mgm. solids) become $620/R$ and $43/R$ respectively, where R = generation (cell-doubling) time in hours. *Nostoc* grows at *ca.* 75% of the control rate when fixed nitrogen is withheld, corresponding to a 10 hr. doubling period. On this basis, Q_{CO_2} is 62.0 and Q_{N_2} is 4.3; similar values were indicated by the growth data of Allen and Arnon for *Anabaena* (1). The maximum rate of gas absorption associated with N_2-fixation in blue-green algae thus is *ca.* 1/15th that of carbon dioxide assimilation; it is too low to be satisfactorily measured manometrically in the dilute cell suspensions required for light saturation (10 mm.^{3} cells/vessel would absorb up to *ca.* 5 mm.^{3} N_2/hour in the absence of combined nitrogen). Absorption of N_2 must be taken into account, however, in two-vessel measurements of *Nostoc* photosynthesis when the cells are grown and tested in nitrogen-deficient media.

Photosynthetic Capacities

Our comparisons of the photosynthetic capacities of the Gerloff strain of *Nostoc muscorum* and the Emerson strain of *Chlorella pyrenoidosa* refer to continuous autotrophic cultures that were maintained for weeks under very similar conditions of growth, the cells being harvested at intervals of one to three days as noted (Tables III, IV). Useful "minimum" values for photosynthetic capacity may be calculated from the growth rates and carbon contents of these algae, but photosynthetic capacity on this basis was regularly lower than the full capacity for photosynthesis under the conditions of the two-vessel method at the same temperature. The carbon contents of *Nostoc* and *Chlorella* grown as described are both *ca.* 50% of the solids. As noted above, their net carbon dioxide assimilation rate during growth is $620/R$ when R = generation (doubling) time in hours, the assimilation rate being expressed as Q_{CO_2} (mm.^{3} CO_2/hour/mgm. solids). Generation times for *C. pyrenoidosa* and *N. muscorum* at 25° C. were typically 6 and 7.5 hours, corresponding to Q_{CO_2} values of 103.5 and 83, respectively. Kratz and Myers (40) found that the optimum temperature for growth of *C. pyrenoidosa* is 25° C., as compared to 32.5° C. for *Nostoc muscorum*, so *Chlorella* is favored by the use of 25° C. The carbon dioxide assimilation rate calculated on a solids basis from the growth rate at 25° C. is 20% lower in *Nostoc* vs. *Chlorella*, a difference which should disappear when each organism is grown at its optimum temperature. Assuming that the carbon content of *Anacystis* (44) is also close to 50% of the solids, its Q_{CO_2} for two-hour doubling periods at 39° C. becomes *ca.* 300, which is similar to its experimentally observed photosynthetic capacity at the same temperature (44).

The temperature used in measuring photosynthetic capacities ideally should be optimal, which might necessitate the use of widely different temperatures on different organisms and tissues. Optimum temperatures are somewhat difficult to define since they are influenced by the duration of the exposure period and by the previous history of the cells, but they usually are well above 25° C. for algae. In our studies, short-term photosynthetic capacities were doubled in *Nostoc* and were increased 50% in *Chlorella* upon raising the temperature from 25° to 35° C. The highest photosynthetic capacities on record are for the blue-green alga *Anacystis* and for a high temperature strain of *Chlorella* which was grown and tested at 40° ± 1° C. (41). Use of 25° C. in Table V allows direct comparisons with the extensive data of Willstätter and Stoll (58) on the photosynthetic capacities of the green leaves of woody and herbaceous plants, as well as with marine algal fronds (36) of which *Ulva* is quite typical (37). The high photosynthetic capacities of blue-green algae are evident at 25° C.; Table V summarizes existing information on this point. Thermophilic blue-green algae display still higher capacities, however, when grown and tested at higher temperatures (44).

On a solids basis, the photosynthetic capacities of our *Nostoc* and *Chlorella* populations were the same when grown and tested at 25° C.; similar photosynthetic capacities on a solids basis were exhibited by *Anabaena* and *Anacystis*

TABLE V

PHOTOSYNTHETIC CAPACITIES OF ALGAE AND GREEN LEAVES AT 25° C., UNDER CONDITIONS OF LIGHT AND CO_2 SATURATION

Plant	Photosynthetic capacity in mm.³ O_2/hour				Reference
	Per mgm. solids	Per mgm. chlorophyll	Per 10^9 cells	Per mm.³ p.c.	
Nostoc muscorum	157–251	9,230–14,770	1,630–2,610	20.4–32.6	This paper
Anabaena variabilis	179–183	—	—	36.9–37.7	(41)
Anacystis nidulans	85–170	2,980–13,400	—	25.2–47.2	(44)
Chlorella pyrenoidosa	161–245	3,980–6,110	780–1,200	39.8–61.1	This paper
Ulva lactuca	25.4	3,490	—	Per mgm. fr. wt. 6.18	(36)
Helianthus annuus	Mm.³ CO_2 65.7–69.8	Mm.³ CO_2 5,550–8,500	—	Mm.³ CO_2/mgm. fr. wt. 11.6–12.7	(58)
Green tree leaves, 25 samples, 7 genera, May–July. Table 22 (58)	Mm.³ CO_2 13.2–53.9	Mm.³ CO_2 2,640–8,150	— —	Mm.³ CO_2/mgm. fr. wt. 3.66–12.3	(58)

populations which were grown at similarly high light intensities (41, 44). The foregoing algae all exhibit higher photosynthetic capacities on a fresh and dry weight basis than marine algal fronds (37) as well as leaves of higher plants (58). The photosynthetic capacity of *Nostoc* and of several *Anacystis* cultures (44) exceeded that of our most active *Chlorella* populations on a chlorophyll basis at 25° C.; the photosynthetic capacity of *Nostoc* on this basis also was regularly higher than that of green leaves (58). The *Nostoc* cells individually were much larger than the *Chlorella* cells used in this study; although *Nostoc* had a much lower chlorophyll content per unit of fresh or dry weight, its chlorophyll content per cell was close to that of *Chlorella*. Photosynthetic capacity per billion cells was regularly much higher in *Nostoc* than in *Chlorella* (Table V). The only basis on which the photosynthetic capacity of *Nostoc* was below that of *Chlorella* at 25° C. was in terms of "packed" cell volume, which was caused by the loose sediments of low solids content formed by *Nostoc* filaments. Among the plant materials represented in Table V, the green leaves of higher plants are morphologically the most highly organized for photosynthesis. The primitive blue-green algae regularly exhibited higher photosynthetic capacities than have been recorded for leaves, in terms of fresh weight, dry weight, and chlorophyll content.

Discussion

The results obtained in the present and related investigations allow several conclusions concerning the photosynthetic characteristics of the filamentous blue-green alga, *Nostoc muscorum*. It is physiologically more sensitive than *Chlorella* in several respects; it requires a more dilute mineral salt solution for its growth, and a more dilute bicarbonate buffer for photosynthesis measurements; it is also injured by slightly acidic as well as by potassium-free media.

Injury of *Nostoc* by a pH of 5.5 suggests that its buffering properties are defective only slightly below neutrality. Injury of *Nostoc* by washing with neutral potassium-free media probably involves altered permeability and loss of cellular constituents as in *Nitella* (46). Semipermeability of *Nostoc* apparently is entirely lost when freshly harvested suspensions are subjected to traces of toluol in the Waring blendor: phycocyanin and chloroplastin are released from *Nostoc* within minutes, but chloroplastin is not released from *Chlorella* under these conditions.

The reduction of photosynthesis in *Nostoc* and of the Hill reaction in isolated chloroplasts by preparatory washing treatments have different causes. Injury of *Nostoc* photosynthesis by washing is a deep-seated phenomenon caused primarily by the absence of potassium, which needs only be present in a few parts per million; potassium does not participate in the Hill reaction (15, 16, 33). The "chloride effect" on washed chloroplasts is anionic and reversible (33); the "potassium effect" on *Nostoc* photosynthesis is cationic and essentially irreversible. The markedly different responses of photosynthesis and the Hill reaction to washing injury indicates that phycocyanin redistribution does not occur.

Respiration in *Azotobacter* is reduced or even eliminated by thorough washing with phosphate buffers, this effect being reversed and prevented by magnesium ions (34). The latter phenomenon is caused by a different cation, and differs in its reversibility from the washing injury to *Nostoc*. Among all the "washing effects" on plant and bacterial cells revealed by a literature survey, the responses of *Nitella* (46) to pure water most closely resembles those we have observed on *Nostoc*. Osterhout obtained evidence of altered semipermeability in *Nitella* that was induced by distilled water, with attending losses of organic constituents; the low reversibility of the potassium effect in *Nitella* and *Nostoc* can be tentatively explained on this basis. An outward traffic of organic products appears to characterize blue-green algae during their normal growth: nitrogenous products have frequently been found in their culture media (31), and blue-green algae (including *Nostoc*) must provide all of the organic food requirements in some related way to their fungal symbionts in lichens.

Photosynthesis in *Nostoc* and *Chlorella* exhibited numerous similarities in this study. Their photosynthetic capacities on a solids basis were very similar, as were their responses to high temperatures (>40° C.). The saturating light intensity requirements and optimum temperatures were very similar; the aerobic induction periods were of the same duration, and the responses to anaerobic conditions and phenylurethane also were similar. Intermediates of carbon assimilation in *Nostoc* were shown to be the same as in *Chlorella* by Allison *et al.* (2, 3) and by Norris *et al.* (45).

Nostoc and other blue-green algae have properties which may be advantageous in mass-culturing (adaptability to high temperatures (10), ability to assimilate molecular nitrogen, and low mineral salt and CO_2 concentration requirements for growth). Although *Nostoc* has numerous distinguishing

characteristics, none of these are directly related to the absence of chloroplasts in this organism. Its high photosynthetic capacity and the similar photochemical efficiencies of *Chroococcus* vs. *Chlorella* (27) show that the absence of organized chloroplasts has little influence on photosynthesis (4). The role of the chloroplast in photosynthesis appears to be similar to that of the nucleus in heredity. The tiny structures which are essential for the expression of heredity are much smaller than the nucleus, but are borne within the nucleus when it is present; the same organisms (blue-green algae, photosynthetic bacteria) which exhibit photosynthesis without chloroplasts exhibit heredity without nuclei.

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THE PEACH REPLANT PROBLEM IN ONTARIO

IV. FUNGI ASSOCIATED WITH REPLANT FAILURE AND THEIR IMPORTANCE IN FUMIGATED AND NONFUMIGATED SOILS[1]

By R. N. Wensley[2]

Abstract

Soil fumigation with MC-2 (methyl bromide) inhibited many of the fungi found in the rhizosphere of peach replants. This chemical, as well as DD (a propane–propene mixture), induced greater growth of peach seedlings in most soils than either Dowfume W40 (ethylene dibromide) or CBP HI 806. Methyl bromide fumigation also permitted second year replants to develop free of root rot. On the other hand, the use of Dowfume W40, which was ineffective against the fungus flora, usually resulted in greater growth than that obtained in check soils. The soil fumigants used did not modify the depressing effect of toxins on the growth of replants in tree sites although this effect gradually diminished with the passage of time. Fourteen species of fungi out of several hundred isolated from the rhizosphere and root tissues of peach replants in fumigated and non-fumigated peach orchard soils were found to be facultative parasites on the roots of peach seedlings when grown under greenhouse experimental conditions. Of these, *Fusarium equiseti*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium solani*, *Alternaria tenuis*, *Myrothecium verrucariae*, and members of Mycelia Sterilia were isolated most frequently while *Papularia sphaerosperma*, *Gliomastix convoluta*, and species of *Coniothyrium*, *Curvularia*, *Humicola*, *Peyronellaea*, and *Truncatella* appeared in the rhizosphere of certain peach trees and not in that of others. The experimental conditions most favorable to the infection of roots by these fungi proved to be low soil moisture combined with high soil temperature.

Introduction

These studies form a part of the co-ordinated investigations of the underlying causes of peach replant failure conducted at the Harrow Science Service laboratory. The present paper deals with a study of the fungi associated with peach orchard soils and the rhizosphere of peach replants, and the influence of soil fumigation with several chemicals on the fungus flora and the growth of young peach trees.

A detailed review of literature pertaining to this problem has been reported by Koch (1) and the existence of toxins in old peach orchard soils resulting from the microbial breakdown of peach-tree bark was proved by Patrick (2).

General Methods and Materials

Root and soil samples used for analyses of the fungus flora in fumigated and nonfumigated soils were selected from locations as widely separated as possible so as to include those in which peach replant failure had occurred as well as those in which the problem was not manifested. The fungus flora associated with peach replants in these soils during the first year in which replant failure was generally most severe was determined both quantitatively and qualitatively. Finally, pathogenicity tests were conducted to determine which fungi

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of the rhizosphere flora caused lesions and browning of peach roots under experimental conditions.

Several fumigants were chosen because of their respective nematocidal and fungicidal properties:

Methyl bromide and its formulation, Dowfume MC-2 (methyl bromide containing 2% chloropicrin)—Dow Chemical Co. of Canada.

Ethylene dibromide and its formulation, Dowfume W40 (41% by weight of ethylene dibromide)—Dow Chemical Co. of Canada.

DD, a propane–propene mixture—Shell Chemical Corporation.

CBP formulation CBP HI 896—Shell Chemical Corporation.

In greenhouse experiments, the dosage of each chemical per 10-in. pot of soil was as follows: methyl bromide (0.8, 1.2 ml.), ethylene dibromide (1.2 ml.), and CBP HI 806 (4.2 ml.). In the field the following dosages were applied: MC-2 (2 lb. per 100 sq. ft.), Dowfume W40 and DD (40 gal. per ac. in 10-in. centers), and CBP HI 806 (32.6 gal. per ac.).

Field fumigation tests were conducted both in tree sites (locations of former peach trees) and in intersites (locations between former tree sites). After different periods of growth in these soils both in the orchard and in greenhouse pots, growth measurements were made of each stem and twig to determine the relative total elongation in each soil. Supplementary evidence of the benefits of fumigants used in these experiments was obtained by a rating of leaf color and size, amount of foliage, and the vigor of peach seedlings.

Experiments and Results

Greenhouse Fumigation Studies

A study was made of the effect of fumigation of soils on the growth of peach seedlings under conditions of low (14% of oven-dry weight) and high (20% of oven-dry weight) soil moisture subsequent to treatment and planting. In this experiment methyl bromide was applied at the rate of 1.2 ml. per 10-in. pot and was compared with an equal dosage of ethylene dibromide in seven soils where moisture was a factor limiting to growth and compared with CBP HI 806 at 4.2 ml. per 10-in. pot in the same soils as well as three additional soils where moisture was not limiting to growth. The soils were obtained from orchards where replant failure had been serious as well as from replantings where failure had not occurred.

Fig. 1 shows that, where moisture was a limiting factor, greater growth occurred consistently in soils treated with methyl bromide than with EDB. Moreover, the increase in growth was greatest in soils where the replant failure had been most severe and least in soils in which replant failure had not occurred. On the other hand, methyl bromide was ineffective in increasing growth in all but one of the same soils when moisture was not a limiting factor in development of the seedlings. This one exception occurred in a soil obtained from an orchard in which replant failure had been severe for a period of six years. Here, fumigation induced more than twice as much growth as in

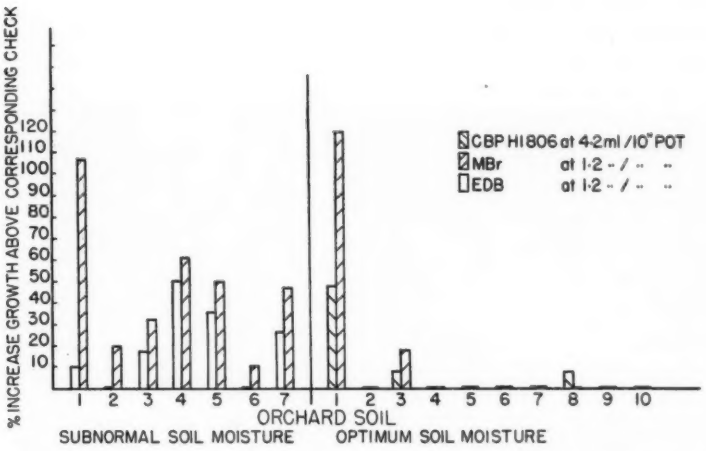

FIG. 1. Percentage of growth increase over corresponding checks of stems and twigs of peach seedlings grown in fumigated soils at low and at optimum soil moisture levels during 86 and 74 days respectively.

corresponding checks. Since, as compared with the growth of nonfumigated check, less growth resulted from fumigation with methyl bromide of soils not previously planted to peaches than of soils in which peach trees had been removed, it was evident that the control of microorganisms favored by preceding sour cherry trees in one orchard and a rotation of miscellaneous crops in another was less important to the growth and health of peach replants than of microorganisms favored by a preceding crop of peaches.

Fumigation of the above soils with ethylene dibromide induced less growth than fumigation with methyl bromide under comparable conditions of low soil moisture. Ethylene dibromide was likewise least beneficial in soil previously planted to a crop other than peaches. Further, CBP HI 806 proved ineffective when moisture was not a limiting factor in all but one soil in which both this material and methyl bromide were beneficial.

FIELD FUMIGATION STUDIES

Soil fumigation experiments with MC-2, Dowfume W40, CBP HI 806, and DD were conducted in the Science Service peach orchard at Harrow and in six commercial orchards in the vicinities of Harrow, Ruthven, and Leamington. In these experiments an attempt was made to evaluate the relative importance of nematodes and fungi in different soils by comparing the growth obtained in soils that were treated with methyl bromide, which possesses good nematocidal and fungicidal properties on the one hand with ethylene dibromide, which was nematocidal but not fungicidal, on the other. The influence of fumigation on the fungus flora in these soils is discussed in a later section of this paper.

Science Service Orchard

Soil fumigation experiments with MC-2 and Dowfume W40 were conducted in this orchard during 1953 and 1954. In the second year, two of the four plots in each of the three original treatments (check, MC-2, and Dowfume W40) were refumigated with MC-2 and the remainder left untreated to determine the residual benefits of both fumigants when the plots were replanted to peach seedlings.

The relative growth of plants in treated and in check plots after 62, 108, and 152 days is shown in Fig. 2. At the end of the 152 day period total growth of seedlings in MC-2 plots was double that in check plots and 1.72 times greater than that in Dowfume W40 plots. These differences were

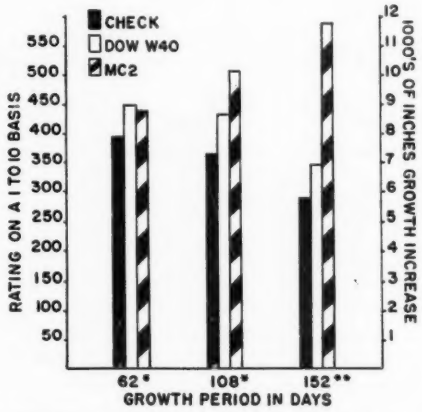

FIG. 2. Growth response of peach seedlings on a 1 to 10 rating basis* and in inches of stem and twig growth increase** in fumigated and nonfumigated soils during several periods of growth in 1953.

slight during the initial 62 days but with the passage of time, the greater benefits of MC-2 fumigation became clearly discernible in terms of the greater volume of foliage and the more normal color and greater size of seedlings in MC-2 plots than in other plots (Figs. 3 to 13). A further examination of these seedlings before their removal in the spring of 1954, 214 days after planting, showed that all seedlings in MC-2 plots were healthy and vigorous while those in check and Dowfume W40 plots were in various stages of degeneration. Additional evidence of the greater residual benefits of MC-2 as compared with Dowfume W40 was obtained from growth data shown in Fig. 15.

FIGS. 3 and 5. End view of row of peach seedlings in check plots. FIGS. 4 and 6. End view of row of peach seedlings in MC-2 and Dowfume W40 plots respectively. FIGS. 7, 8, and 9. Side view of row of peach seedlings in MC-2, Dowfume W40, and check plots respectively. FIGS. 10, 11, and 12. Relative growth of peach seedlings in MC-2, Dowfume W40, and check plots respectively. FIG. 13. Relative root and stem development of peach seedlings in A (MC-2), B (Dowfume W40), and C (check) plots. Period of growth for the above, 108 days.

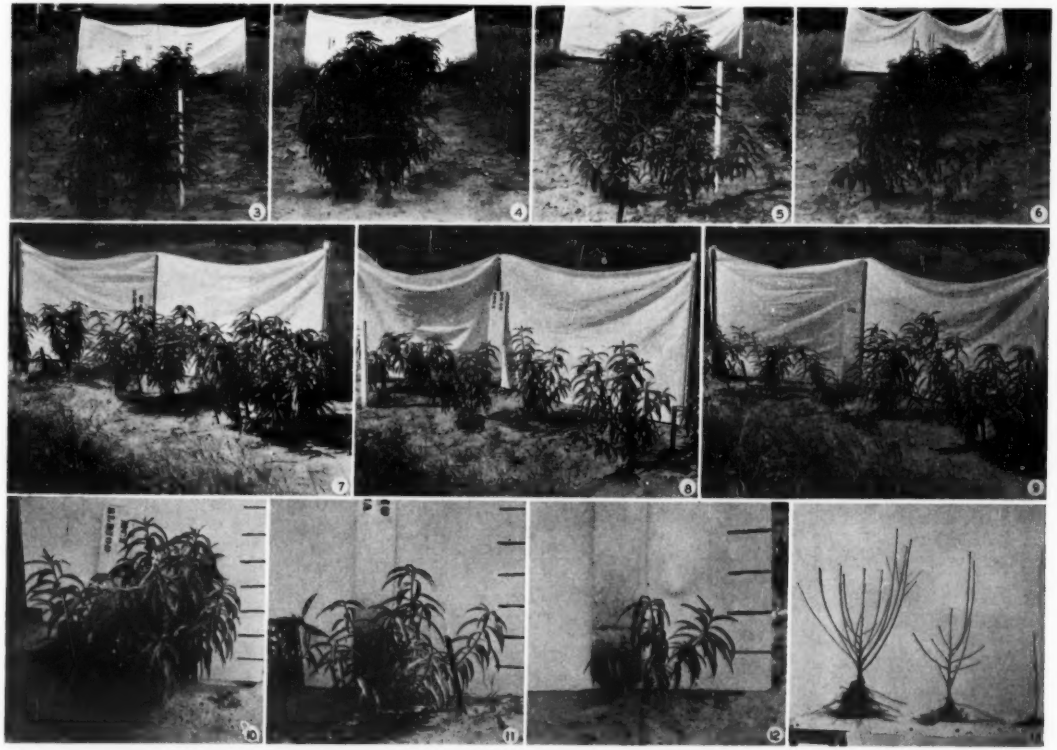

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PLATE II

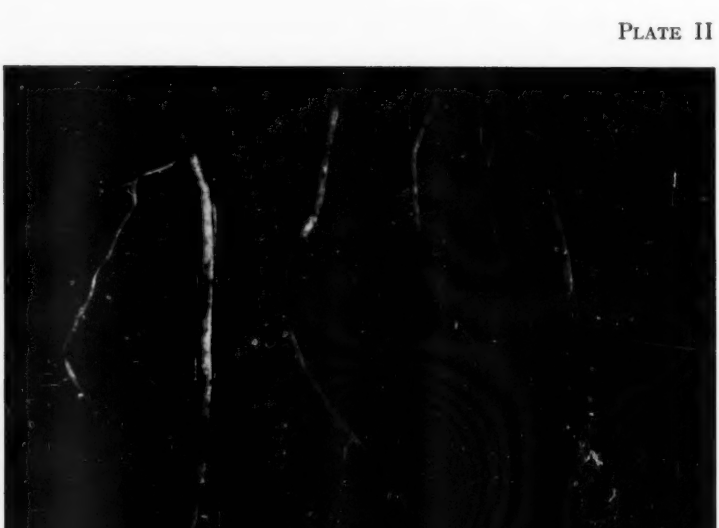

FIG. 14. Root necrosis caused by *Fusarium moniliforme*.

It was shown that the growth made by seedlings planted in 1954 in plots fumigated with MC-2 during the previous spring was nearly equal to that made in MC-2, check, and Dowfume W40 plots which were refumigated with MC-2. The fact that growth in MC-2 plots at the end of two years approximated closely that which was made in refumigated MC-2 plots indicated that MC-2, either directly or through an induced change in the microbiological balance in the fumigated soils, inhibited harmful organisms in this orchard to a point at which their populations were relatively innocuous during a two-year period. Otherwise, retarded growth and other symptoms of replant failure comparable to that in untreated soil would be expected as a result of attack by parasitic organisms.

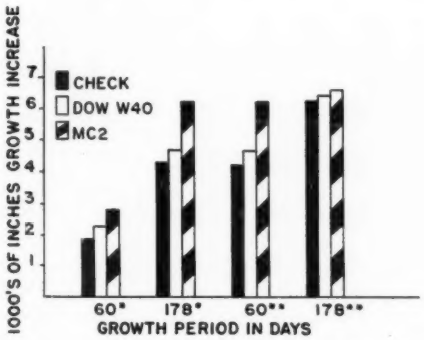

FIG. 15. Growth increase of peach seedlings in soil receiving** or not receiving* a superimposed MC-2 fumigation in 1954.

Tree Site (Toxin) Effect

Data in Fig. 16 show the effect of toxins, which have been proved by Patrick (2) to be HCN and benzaldehyde and which are present in former tree sites, on the growth of peach replants during the second and third years after removal of the old peach orchard. Evidence of a toxic effect on growth in former tree sites is also shown in Figs. 3 to 9 where growth is seen to diminish as the distance of the new tree location decreased from the intersite toward the center of the former tree site. The importance of toxins to replant failure in tree sites of this orchard is indicated by retarded growth that averaged 40% less than that in adjacent intersites in the second year and 11% in the third year after the removal of the old peach trees. During the first two years, therefore, the harmful effect of toxins on replants alone was great enough to account for replant failure in the Science Service orchard. However, this toxic effect did diminish with the passage of time and was relatively slight during 1954. In both 1953 and 1954 the difference in magnitude of the toxic effect on growth in fumigated and nonfumigated soil was slight and it must be presumed that fumigation did not modify the toxic factors present at the time of treatment.

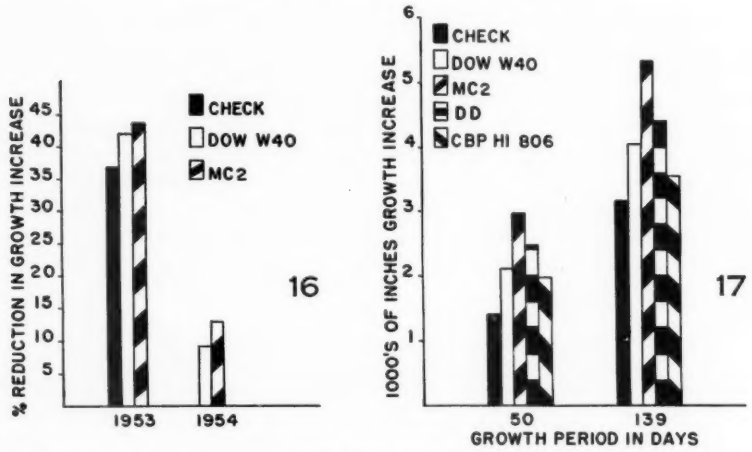

FIG. 16. The average percentage reduction in growth of peach seedlings attributable to factors uninhibited by soil fumigation in former tree sites in 1953 and 1954.

FIG. 17. Stem and twig growth increase of peach seedlings in six replantations after soil fumigation, during 50 and 139 days of growth.

District Orchards

Soil fumigation in these orchards was restricted to intersites in order to avoid the influence of toxic factors causing tree-site effect on growth and to investigate instead the effects which modification of the soil microflora might have on the growth of peach seedlings. Replant failure had not been equally severe in these soils, which differed in type, moisture holding capacity, and drainage, as well as in crop histories. However, all orchards conformed to the requirement of having old trees removed shortly before fumigation.

Data in Fig. 17 show that greater growth was made by peach seedlings in soil fumigated with MC-2 than in check soils and soils fumigated with DD, Dowfume W40, and CBP HI 806. The latter fumigants ranked next, in the order given. Both MC-2 and DD induced greater growth than was obtained in corresponding checks in all orchards. On the other hand, Dowfume W40 and CBP induced greater growth in some soils and not in others. These results indicated that MC-2 and DD were more broadly applicable than Dowfume W40 and CBP under different soil conditions which influence the microflora associated with replant failure.

Growth response to fumigation was not uniform between the several orchards and, as might be expected, varied with differences in the microflora associated with different soil types and conditions. However, the physical differences of these soils did not alter the relative effectiveness of the several fumigants used.

The importance of inhibiting microorganisms that attack the new roots of peach replants was emphasized both by the greater daily growth in fumigated than in nonfumigated soil during the initial 50 days and the final total growth

in effectively fumigated soils. Methyl bromide was most effective in both respects. It is assumed that the normal growth of roots through soil in which harmful organisms had been suppressed by fumigation would be an important factor in the development and survival of peach replants.

The possibility that a part of the growth response induced by methyl bromide was attributable to a stimulation of the plant itself was studied under greenhouse conditions. Each of two fumigated soil series, one of which was steam-sterilized before fumigation, and two check series were planted to 15 peach seedlings each. Growth measurements were made at the end of 30 days. The resulting data showed that there was no response by peach seedlings to methyl bromide in steam-sterilized soil. Furthermore, the aggregate growth in the steam-sterilized soil that was fumigated with methyl bromide was less than that obtained in soil that was only steam-sterilized. It was evident from these data that the benefits of fumigating infested soils with methyl bromide were the result of its inhibition of microorganisms in the soil and not of direct stimulation of plant growth by the chemical.

Further data obtained from a field experiment did show, however, that methyl bromide may be phytotoxic to peach replants under certain soil conditions. The normal rapid penetration of and disappearance from the soil by methyl bromide was interfered with in this experiment by low temperatures and heavy precipitation which occurred during the 10 day waiting period. The toxic effect of methyl bromide under these adverse conditions was manifested first by sparse foliation and later by a 55% mortality among trees planted in fumigated soil. All trees in nonfumigated soil survived. This high mortality among peach replants emphasized the hazards of fumigation under certain unfavorable conditions and the advisability of practicing fumigation in the fall. The latter practice would eliminate losses caused by too short a waiting period and would also avoid delays that occur in the spring because of unfavorable weather.

Fungi Associated with Replant Failure in Fumigated and Nonfumigated Soils

A comprehensive survey of fungi associated with peach replants in fumigated and nonfumigated soils was made in conjunction with soil fumigation investigations reported in this paper. The fungus flora of rhizosphere and soil samples were analyzed quantitatively and qualitatively by the dilution method. In addition, fungus isolates were obtained by plating diseased root tissues on the following media: a 2% potato dextrose agar, an oatmeal agar and an enriched medium of agar (20 gm.), dextrose (10 gm.), $NaNO_2$ (2 gm.), K_2HPO_4 (2 gm.), potato extract (10 ml.), yeast extract (2 gm.), malt (2 gm.), and soil extract (500 ml.) with sufficient water to make up 1 liter. Bacteria and actinomycetes that were active in the experiment were suppressed on plates with rose bengal (1 : 15,000) to permit the analysis of residual fungi.

In the Rhizosphere of Replants (Science Service Orchard)

Many fungi were encountered in the analyses of the rhizosphere flora of peach replants in fumigated and nonfumigated soils. Some of these were widely distributed and common to peach rhizosphere while others occurred occasionally in low concentration in one or more of the orchards throughout southwestern Ontario. The distribution of these fungi in fumigated and nonfumigated soils is shown in Table I.

Eleven of the 53 isolates listed in Table I were found commonly in the rhizosphere of seedlings growing in nonfumigated and ineffectively fumigated soils while only five appeared commonly where effective fumigation with methyl bromide was practiced. At the same time 33 of the fungi found either occasionally or frequently in nonfumigated and ineffectively fumigated soils were absent in the rhizosphere of seedlings planted in soils that had been fumigated by methyl bromide. In addition to these qualitative modifications of the rhizosphere fungus flora, the relative abundance of each surviving fungus was altered. These changes consisted primarily of increased populations of several fungi, including species of *Hormodendron*, *Mucor*, and *Penicillium*, while *Trichoderma viride* and *Myrothecium verrucariae* were slightly suppressed.

In the greenhouse, species of *Penicillium*, *Mucor*, *Trichoderma*, and *Fusarium* predominated in the rhizosphere of seedlings planted in seven fumigated and nonfumigated orchard soils but no distinct pattern of occurrence was discernible within the period of examination. However, fumigation with methyl bromide did reduce the number of species present in these soils, and, with the exception of a species of *Mucor*, a species of *Penicillium*, *Myrothecium verrucariae*, and *Trichoderma viride*, the population of each was reduced below that in nonfumigated soils. Of the 18 genera and 34 species the most frequently occurring were four species of *Fusarium* (*F. equiseti*, *F. moniliforme*, *F. oxysporum*, and *F. solani*), 11 species of *Penicillium*, and *Trichoderma viride*. Species of *Aspergillus*, *Gliocladium*, *Oospora*, *Rhizopus*, *Zygorrhynchus*, *Acremoniella*, and *Chaetomium* occurred infrequently and generally in low concentrations. *Metarrhizium anisopliae* (Metsch.) Sorok., which was originally isolated from an insect, was obtained from only one of the seven rhizosphere sources in this experiment but later it was isolated from the rhizosphere of seedlings which were severely diseased in the Harrow Science Service orchard. In the latter instance, the large populations of black peach aphids (*Aphis persicae-niger*) present on the replants suggested a relationship between the above fungus and aphis.

Analyses were made of the rhizosphere fungus flora of peach seedlings growing in soils that had been fumigated by methyl bromide and in nonfumigated soils in the Harrow Science Service orchard 108 days after planting. At this time numerous replants in nonfumigated soil were either dead or dying and their root systems were severely stunted as a result of root rot. New growth was limited to short rootlets of which many had become lesioned and unhealthy shortly after their appearance. In soils that had been fumigated

TABLE I

FUNGI ISOLATED FROM PEACH RHIZOSPHERE IN FUMIGATED AND NONFUMIGATED SOILS

Fungi	Distribution in soils		
	Fumigated		Nonfumigated
	MC-2	Dow W40	(CK)
*Alternaria tenuis**	×	×	×
Coniothyrium sp.*		×	×
Curvularia sp.*		×	×
*Fusarium equiseti**	×	×	×
*Fusarium moniliforme**	×	×	×
*Fusarium oxysporum**	×	×	×
*Fusarium solani**	×	×	×
*Gliomastix convoluta**		×	×
Humicola sp.*		×	×
*Myrothecium verrucariae**	×	×	×
*Papularia sphaerosperma**		×	×
Peyronellaea sp.*		×	×
Mycelia Sterilia*	×	×	×
Truncatella sp.*		×	×
Acremoniella sp.		×	×
Aspergillus niger	×	×	×
Aspergillus ochraceus	×	×	×
Aspergillus terreus	×	×	×
Aspergillus ustus	×	×	×
Cephalosporium sp.	×	×	×
Chaetomium globosum	×	×	×
Chaetomium cochliodes		×	×
Cladosporium herbarum		×	×
Clasterosporium sp.		×	×
Coremium sp.		×	×
Fusidium sp.		×	×
Gliocladium spp.	×	×	×
Hormodendron cladosporioides	×	×	×
Hormodendron hordei	×	×	×
Hyalopus sp.		×	×
Hyphoderma sp.		×	×
Isaria sp.		×	×
Metarrhizium anisopliae		×	×
Monilia sp.		×	×
Mucor spp.	×	×	×
Oospora lactis		×	×
Paecilomyces sp.		×	×
Penicillium luteum		×	×
Penicillium restrictum		×	×
Penicillium spp.	×	×	×
Periconia sp.		×	×
Perisporium vulgare		×	×
Phycomycete	×	×	×
Pyrenochaeta sp.		×	×
Rhizopus nigricans	×	×	×
Sordaria hypocoproides		×	×
Spicaria violaceae		×	×
Sporotrichella sp.		×	×
Stysanus medius		×	×
Trichosporium sp.		×	×
Trichoderma viride	×	×	×
Unknown		×	×
Zygorrhynchus sp.		×	×

*Fungus pathogenic on the roots of peach seedlings.

by methyl bromide, however, seedlings were thrifty and had extensive root systems made up of many fine feeding laterals that were free of root lesions.

The most striking difference between the fungus flora of the rhizosphere in nonfumigated and fumigated soils was the greater number of genera and species represented in the former than in the latter rhizosphere. Nineteen genera and 29 species of fungi were isolated from diseased roots in nonfumigated soils compared with eight genera and nine species in fumigated soil. *Trichoderma viride*, *Myrothecium verrucariae*, *Hormodendron cladosporioides*, *Gliomastix convoluta*, and nine species of *Penicillium* comprised the greatest concentration of fungi in the rhizosphere of diseased roots while the remaining species comprised approximately 24% of the population. Among the latter were included *Alternaria tenuis*, *Chaetomium globosum*, *Metarrhizium anisopliae*, *Stysanus medius*, *Spicaria vilaoceae*, and species of *Mucor*, *Humicola*, *Monilia*, *Sporotrichella*, *Aspergillus*, *Cephalosporium*, and *Fusarium*. Few of these fungi appeared in the rhizosphere of seedlings grown in soil fumigated with methyl bromide. The most striking changes in the fungus flora consisted of a reduction of *Trichoderma viride* and *Myrothecium verrucariae* populations and the elimination of all but one species of *Penicillium*, which increased in numbers along with a species of *Mucor* and *Hormodendron cladosporioides*. The latter three isolates comprised 70% of the colonies isolated from the rhizosphere in fumigated soil as compared with 15% in nonfumigated soil.

In the Rhizosphere of Replants (District Orchards)

Quantitative data obtained from rhizosphere analyses made 58 and 86 days after planting are recorded in Table II. These data show that the number of species isolated and the total number of fungi were less in the rhizosphere of seedlings growing in soil that had been fumigated with MC-2 than in the rhizosphere of seedlings growing in nonfumigated soil. In fumigated soils

TABLE II

FREQUENCY OF OCCURRENCE OF RHIZOSPHERE FUNGI IN FUMIGATED (MC-2) AND NONFUMIGATED (CK) PEACH ORCHARD SOILS

	Days of growth							
	58				86			
	Treatment							
	CK		MC-2		CK		MC-2	
Replantation	Spp.*	Col.†	Spp.	Col.	Spp.	Col.	Spp.	Col.
1					16	27	1	21
2	18	62	8	17	19	38	0	0
3					17	58	2	21
4					28	40	6	7
5					11	59	2	18
6					16	22	1	1

**Number of species isolated.*
†Number of colonies of all isolates.

the rhizosphere fungi were reduced generally to one or two species of *Mucor*, *Trichoderma*, and *Penicillium* while in nonfumigated soils Mycelia Sterilia and species of *Fusarium*, *Alternaria*, *Aspergillus*, *Coniothyrium*, *Curvularia*, *Gliomastix*, *Humicola*, *Penicillium*, *Myrothecium*, and *Truncatella* and others predominated in the rhizosphere. This disturbance of the microbial equilibrium and the reduction of the fungus flora within the rhizosphere of replants in soils that were fumigated with MC-2 were accompanied by a reduction in root rot and a seemingly uninhibited development of roots in the fumigated zones. Whether or not these phenomena were attributable to the suppression of fungi as primary or secondary parasites, the fact remains that the greatest numbers of fungi within the rhizosphere were obtained in nonfumigated replantations where replant failure was most severe.

In the Soil

A survey of the fungi associated with soils in old peach orchards, young peach replantations, and nurseries showed that a greater number of genera were present in nursery soils than in most established orchards and replantations. However, species of *Penicillium*, *Mucor*, *Fusarium*, *Alternaria*, *Aspergillus*, *Coniothyrium*, *Myrothecium*, *Trichoderma*, and *Gliomastix*, which were common in orchard and replantation soils, also occurred commonly in the rhizosphere flora of peach replants. Other fungi, which occurred occasionally in some soils and in small numbers, included species of *Chaetomium*, *Clasterosporium*, *Coremium*, *Fusidium*, *Gliocladium*, *Paecilomyces*, *Periconia*, *Perisporium*, *and Sordaria*.

Pathogenicity Experiments

Method

The significance of many fungi that become associated with the rhizosphere of peach replants in infested soils was studied by determining the potential pathogenicity of these fungi on peach seedlings under greenhouse conditions. Numerous isolates from different sources, including mildly and severely diseased replants in both sites and intersites of each orchard and replantation were selected for testing in steam-sterilized and nonsteamed soils by the following split-root technique.

Plywood boxes, 4 in. × 5 in. × 3 in. in dimension, were divided into either two or four chambers by aluminum foil or cardboard partitions. Young peach seedlings of uniform age and size were then so placed that approximately one-half of a root system fell into one soil chamber and the second half into another. Inoculum of the test organism was applied to the root portion in one chamber only. The second half was retained as a check. Contamination of roots retained as check was avoided by burying this half of the root system in soil before inoculum was added in the second chamber. At the termination of seven to eight days, inoculated and uninoculated root portions of each plant were examined for lesions and the severity was noted in each case. Inoculum was prepared and applied in two ways. In the first, inoculum grown on a 2% PDA medium was applied directly at several points along the length

of each root. By this method the capacity of a fungus to parasitize peach roots was determined in each of the areas to which inoculum was applied and the limitation of attack to this area or its spread along the root could be observed readily. The second method consisted of growing each test organism on a 2 : 1 : 2 mixture of sterilized oats, wheat, and vermiculite, respectively, for a period of 10 to 14 days before measured portions of inoculum were applied to the soil. This seeded soil was then covered and set aside for four days before peach seedlings of uniform size were planted.

The Disease Potential of the Fungus Flora in the Rhizosphere of Peach Roots

While most of the several hundred fungi isolated from the rhizosphere of peach roots were incapable of causing root rot of peach seedlings even under favorable greenhouse conditions, the following were moderately to weakly pathogenic; *Fusarium equiseti*, *F. moniliforme*, *F. oxysporum*, *F. solani*, *Alternaria tenuis*, *Gliomastix convoluta*, *Myrothecium verrucariae*, *Papularia sphaerosperma*, species of *Coniothyrium*, *Humicola*, *Peyronellaea*, *Truncatella*, *Curvularia*, and Mycelia Sterilia. None of these isolates was strongly virulent and all were incapable of killing the host when it was grown under favorable experimental conditions. However, in dry soils at temperatures of 70° and above the severity of necrosis increased from slight to severe and was either restricted to the inoculated areas or extended along a part of the root length (Fig. 14). Of the numerous isolates of *Fusarium* that were obtained from peach root rhizosphere in all peach orchard soils, *Fusarium equiseti*, *F. oxysporum*, *F. moniliforme*, and *F. solani* were consistently, if but moderately, pathogenic. Lesions caused by these and other isolates were not only defined but usually extended several centimeters beyond the inoculated area in the destruction of the epidermal and cortical tissues. *Humicola*, on the other hand, caused root rot and browning that was restricted to the inoculated areas while several fungi produced only a generalized browning of roots.

The virulence of pathogenic fungi listed in Table I was not of a high order and no single fungus would be regarded as a serious menace to young peach trees if growing conditions were favorable. It was apparent that these potential pathogens comprised a group of facultative parasites which normally coexisted and competed with other saprophytic fungi within the rhizosphere but which might be stimulated to parasitic activity as a result of changes within the rhizosphere or of decreased resistance of the host.

Effect of Soil Temperature and Moisture on Growth of Peach Seedlings and on the Parasitism of *Fusarium moniliforme*

Moisture and temperature changes are considered to be important among the environmental changes that affect the activities of organisms within the soil. The effect of these two factors on growth of peach seedlings and on the severity of root rot caused by *Fusarium moniliforme* was studied under controlled conditions in the greenhouse. The above fungus selected for this

study was isolated from the rhizosphere of diseased peach roots and was shown to be pathogenic to roots of peach seedlings under experimental conditions (Table I). Measured portions of inoculum in aqueous suspension were added to the soil four days before planting. Peach seedlings were planted in two series of soils, one at low (14% of oven-dry weight) moisture and the other at high (20% of oven-dry weight) moisture and at temperatures of 50°, 60°, 70°, 80°, and 90° F. The severity of disease of each plant caused by *F. moniliforme* was rated on a 1 to 10 basis and the sum of these ratings comprise the aggregate disease rating of the fungus in each treatment shown in Fig. 19. Fig. 18 shows the growth in inches of peach seedlings in each treatment.

Peach seedlings made most growth at 50° F. irrespective of whether moisture was or was not a factor (Fig. 18). While a change in temperature from 50° F. to 60° F. at both levels of moisture caused only a slight reduction in growth, this reduction became pronounced above 60° F. when moisture was a limiting

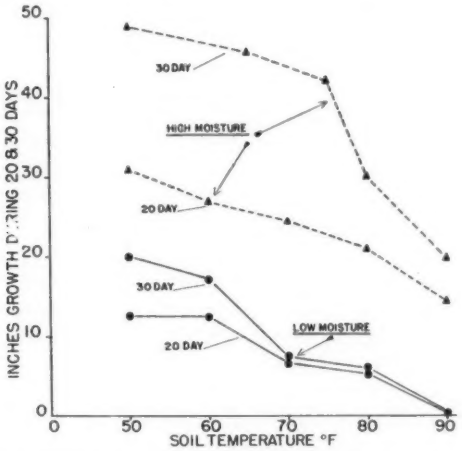

FIG. 18. Growth response of peach seedlings at high and low soil moistures and five soil temperature levels.

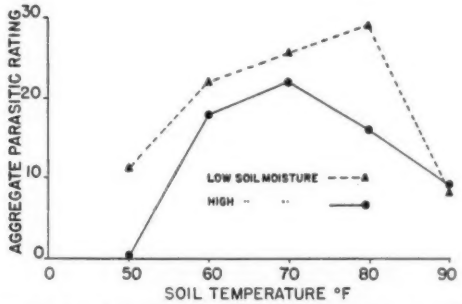

FIG. 19. Effect of soil moisture on parasitism of roots of peach seedlings of *Fusarium moniliforme* at five temperatures.

factor. When the moisture supply was low, little growth was made at temperatures of 70° F. and above, whereas vigorous growth continued when moisture was not limiting. However, at 80° F. and above, growth decreased sharply in both series, although at 90° F., growth of plants in soil which had received adequate moisture was still equal to that obtained in dry soil at 50° F.

Disease incidence likewise increased with a rise in temperature above 50° F. (Fig. 19). Least evidence of parasitism by *F. moniliforme* occurred at 50° F. when moisture was not a factor limiting to growth and the most at 80° F. when the supply of moisture was low. The parasitic activity of the fungus increased sharply between 50° F. and 60° F. at both soil moisture levels and continued to increase at uniform rates up to 70° F.

These data showed that, while a rise in temperature from 50° F. to 60° F. caused the least reduction in growth of peach plants, it caused the greatest increase in root rot at both levels of moisture. Further, a rise in temperature from 60° F. to 70° F. caused the greatest reduction in growth of plants when moisture was a limiting factor, while disease-incidence increased uniformly in both soils.

Maximum growth in the field may be expected, therefore, at temperatures of 50° F. to 70° F. if moisture is not a limiting factor. Otherwise, most growth would occur at temperatures of 50° F. to 60° F. and would decrease at higher temperatures. Within the temperature-range of 60° F. to 70° F., the greater prevalence of root rot caused by *F. moniliforme*, together with the reduction in growth as temperature increased, might contribute to replant failure.

It was previously pointed out that *F. moniliforme* and other facultative parasites that were shown to be pathogenic on roots of peach plants were not strongly virulent and were regarded as secondary contributors to peach failure. However, their contribution and that of other primary parasites, such as nematodes, under conditions of temperature and moisture that were found unfavorable to the host, could determine the extent of replant failure.

Discussion

A greater growth of peach seedlings and a striking reduction of fungus populations, both saprophytes and facultative parasites, within the rhizosphere were produced by fumigating infested soils in which replant failure had occurred. The degree to which fumigation increased growth and reduced microbial populations varied from soil to soil according as the disease expression was mild or severe.

Experiments in the field showed that methyl bromide, and DD to a lesser extent, produced greater growth of peach replants in all soils while Dowfume W40 and CBP were efficacious in some soils and not in others. Further, it was shown that methyl bromide fumigation effectively protected peach replants from disease during a period of two years, which is regarded as sufficient for the successful establishment of a peach replantation.

Although the growth response of peach seedlings attributed to either a direct or induced change in the microbiological balance by soil fumigation

was equally great in intersite and tree-site locations, in no instance did fumigation reduce the depression of growth caused by toxins (Patrick) in tree-site locations. In fact, the reduction in growth ascribed to toxic HCN and benzaldehyde as decomposition products of amygdalin, which may comprise as much as 5% of the dry weight of peach roots, was equally great in fumigated and nonfumigated tree sites. However, this toxic effect did diminish with the passage of time and was shown to vary from site to site according as the waiting period between the time of removal of old trees and the time of replanting was short or extended. In view of these findings it remains advisable, when cultural practices do not permit long waiting periods before replanting, to offset replants from old tree sites to intersite locations.

The reduction of fungus populations that became associated with the rhizosphere of peach replants in nonfumigated soils was considered important to the extent that the following facultative parasites were inhibited by soil fumigation: *Fusarium equiseti*, *F. moniliforme*, *F. oxysporum*, *F. solani*, *Alternaria tenuis*, *Myrothecium verrucariae*, *Gliomastix convoluta*, *Papularia sphaerosperma*, species of *Coniothyrium*, *Humicola*, *Peyronellaea*, *Truncatella*, *Curvularia*, and Mycelia Sterilia. It was shown that fumigation of soils with methyl bromide not only produced greater root development and freedom from root rot than did Dowfume W40 but also reduced the numbers of or inhibited those fungi that were pathogenic on peach roots under experimental conditions. Wherever fumigation failed to support vigorous plant growth, the poor plant growth was associated with inadequate root development and lesioned roots colonized by a large number of fungi. This occurred in some soils that were fumigated with Dowfume W40 but not in soil fumigated with methyl bromide. However, Dowfume W40 was quite effective in some soils and, in the light of its high nematocidal and low fungicidial attributes, this strongly suggests that in such cases nematodes were a primary contributing factor to the failure of peach replants. The evidence as a whole could well be taken to suggest that the combined action of nematodes and facultatively parasitic fungi of a low level of virulence will provide the ultimate answer to the residue of the peach replant problem which is not explained by the action of toxic soil residues already elaborated (2).

Acknowledgments

The writer wishes to express his thanks to Dr. L. W. Koch for his helpful advice and encouragement throughout these studies. I am also indebted to Dr. R. F. Cain, Toronto, Dr. J. W. Groves, Ottawa, and Dr. W. L. Gordon, Winnipeg, for identification of fungi.

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A FURTHER STUDY ON THE GROWTH OF BRASSICA ROOTS IN SOLUTIONS OF PECTIC ENZYMES[1]

By R. G. H. Cormack[2]

Abstract

This paper describes the action of the enzyme preparation Pectinase on the epidermal cells of living roots of *Brassica* seedlings. The striking results obtained are attributed to the effective but non-toxic properties of the preparation and to better fungal and bacterial control.

Introduction

A preliminary paper (8) described the effects of growing *Brassica* seedling roots in a solution of pectic enzymes, Pectinol 100-D. These experiments were undertaken in an attempt to explore further the chemical nature of both epidermal and root-hair cell walls. The most striking effects produced by Pectinol 100-D were the repression of hairs, the separation of epidermal cells, and increased sloughing of rootcap tissue. The separation of the epidermal cells was such that it could be explained on the basis of enzyme action on the outer cementing layer, which left the inner cellulose layer intact.

Although the results obtained with Pectinol 100-D were conclusive, growth of the roots took place only in flowing solutions; even then growth was poor and the roots themselves had a discolored and unhealthy appearance. It seemed worthwhile to experiment with another kind of pectic enzyme preparation which would be less injurious than Pectinol 100-D and yet produce the same effects. The present paper describes experiments with Pectinase, a product of National Biochemicals Corporation, Cleveland, Ohio. According to Sumner and Somers (14) the enzyme Pectinase acts on soluble pectin, calcium pectate, and pectic acid. The present paper describes also refinements in technique designed to reduce fungal and bacterial infection.

Materials and Methods

Farr (10), at the beginning of a long series of experiments on the elongation of root hairs, selected roots of *Brassica* seedlings for his experimental material because they produce abundant hairs in tap water. During the course of studies on the development of root hairs, the present writer (4–8) found seedling roots of several *Brassica* species, Georgia collards, cabbage, white mustard, and rape most satisfactory. A special feature of *Brassica* roots is the differentiation of the epidermis into alternating rows of short and long cells (4, 6, 7). Normally the short cells produce the hairs, while the long cells remain hairless. The method outlined in this report is essentially the same as that reported earlier (8) with only minor changes in technique.

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Contribution from the Department of Botany, University of Alberta, Edmonton, Alberta.
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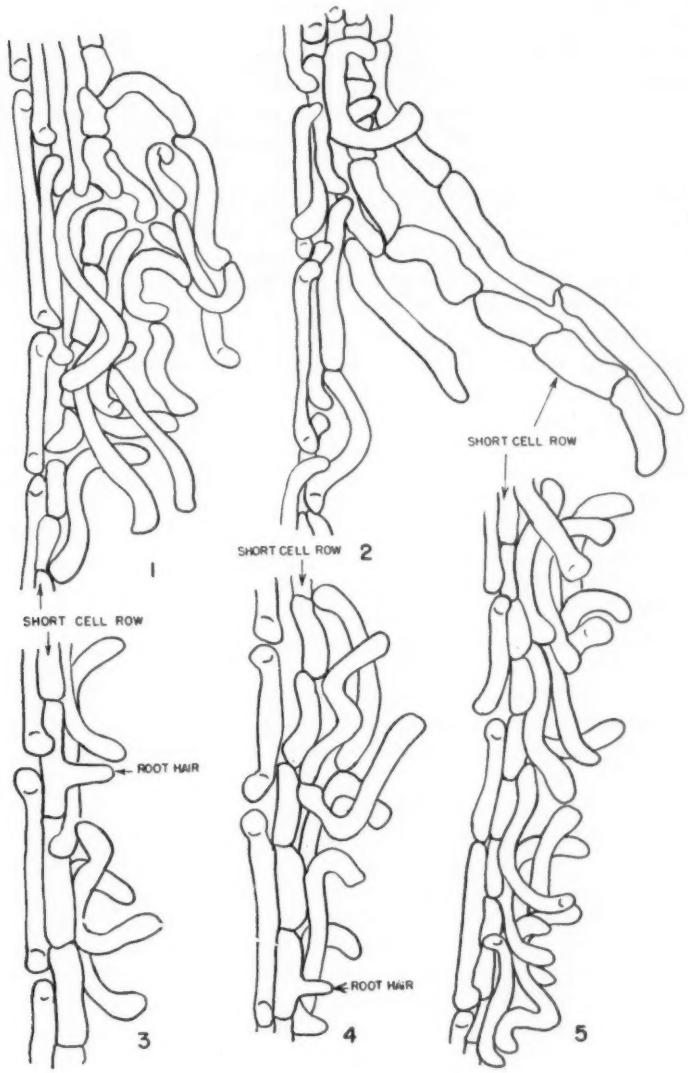
The procedure was as follows. Rape seeds (*Brassica napus* var. *oleifera*) were dusted with Orthocide to prevent fungal growth and placed on moist filter paper in Petri dishes to germinate. Following germination, when they had reached a length of 2–3 mm., the roots of a dozen seedlings were placed through small holes in pieces of stiff, paraffin-coated paper and floated in boiled tap water in small beakers. Eight hours later the floats were transferred to a filtered solution of Pectinase at which time the roots all showed an increase of 2–3 mm. in length. Various concentrations were tried, but the best results were obtained in a 1% solution of Pectinase in distilled water.

At the end of 16 hr., when the roots measured from 10 to 25 mm. in length, they were removed from the solution and examined under the microscope. The usual procedure was to examine only half of the roots and to transfer the float bearing the remainder to a beaker containing a saturated solution of calcium sulphate or tap water.

During the investigation both bacterial and fungal infections were found to be detrimental to root growth particularly at higher temperatures that favor enzyme activity. Orthocide inhibits the growth of fungal organisms but does not restrict the spread of bacteria. Fairly good bacterial control was obtained by keeping the temperature constant at 24° C. and by adding small amounts of streptomycin (Streptomycin Sulphate, Merck & Co.) to the Pectinase solution. Although streptomycin is known to be highly toxic to higher plants (1, 2), the addition of two to five drops of 1% streptomycin sulphate to 50 cc. of the Pectinase solution gave good protection and at the same time did not hinder growth of the roots or enzyme activity. When these precautions were observed seedling roots were found to grow equally well in both standing and flowing solutions.

Observations and Conclusions

Roots grown in a 1% standing solution according to the method described above were healthy and only faintly discolored. When whole roots were examined under the microscope they showed two distinct regions—a short, upper normal region and below this a much longer deformed region. The upper region was developed in tap water prior to immersion of the roots in the Pectinase solution. It was covered with root hairs and was not affected by the solution. The lower region, which had developed in Pectinase only, was deformed and characterized by the lack of hairs and by a marked separation of the epidermal cells. Individual variations occurred but the arrangement and shape of epidermal cells were greatly altered in every root. The bizarre appearance of the epidermis is shown in the camera lucida drawings (Figs. 1–5), and is typical of the entire deformed region. All the drawings were made from whole roots and show only a few rows of cells for a short distance along one side, where they protruded beyond the common surface of the root. The long cells were found to be variously separated from each other, and assumed all manner of peculiar shapes (Figs. 1–5). There were many instances where they had come away completely from the root either as single



FIGS. 1-5. A few rows of epidermal cells showing abnormalities produced in a standing 1% Pectinase solution. The apex of the root is toward the bottom of the page in each figure. × 100

FIGS. 1 and 2. Separation of both long and short cells.

FIGS. 3, 4, and 5. Separation of the long cells only, the short cells maintaining their normal arrangement in long rows.

FIGS. 3 and 4. Production of a short root hair by an occasional short cell.

cells or chains of cells, leaving short gaps or long narrow grooves between the short-cell rows. The short cells were least affected and except in those places where deformity was most marked (Figs. 1, 2), they retained their normal shape and arrangement in even rows (Figs. 3, 4, 5). Only occasionally did the short cells form short hairs (Figs. 3, 4). The walls of both short and long cells were thin but firm and gave a clear-cut test for the presence of cellulose with chlorzinc iodine.

These effects of Pectinase on growing epidermal cells are similar to those previously described for Pectinol 100-D (8), but because of better growth of the roots in Pectinase they are more striking. In these experiments with living seedling roots, why one enzyme preparation should be less toxic and yet more effective than another is not known.

That the roots were healthy and still living despite great deformity was evident by the vigorous growth of Pectinase-treated roots when they were transferred to a saturated solution of calcium sulphate or to tap water. The short cells reacted almost immediately to the improved conditions and in the region of new growth began to form long hairs. The long cells, however, reacted more slowly and were often swollen and separated for some distance in the region of new growth. Eventually deformities ceased, and the root surface assumed a normal appearance. As in previous recovery experiments with ammonium oxalate (4) and Pectinol 100-D (8), that part of the root which had developed in the Pectinase solution remained hairless and completely unchanged on transfer of roots to the calcium solution.

Pectinase had the same action on the root cap as Pectinol 100-D (8), but the effects were more pronounced. They were characterized by maceration of the cells and by the partial or complete dissolution of the cell walls. In some of the cells nothing but the protoplasts remained. Transfer of Pectinase-treated roots to a calcium solution resulted in the development of normal rootcaps.

The results obtained with Pectinase in the present study prove without doubt the occurrence of an outer pectic lamella in the epidermal cell walls (4, 7, 12, 13). They also confirm earlier observations (4, 5, 6) of differences in reaction between the short and long cells. An obvious explanation for the deformities produced by Pectinase is that the enzyme dissolves pectin and pectic acid in the outer lamella while the cells are still elongating and thus prevents the normal change to calcium pectate. In the case of the long cells the solution dissolves the pectic material almost as soon as it is deposited, and, with little or nothing to hold them together, the long cells separate. Only rarely does the solution prevent calcium pectate reaction under the more favorable conditions presented by the short cells (4, 6, 7). As a result the short cells, with few exceptions, do not separate but maintain their normal arrangement in even rows. However, only occasionally do they form hairs. The observations made on recovered roots after transfer to a calcium solution demonstrate once more the importance of calcium for normal root growth and root-hair development (3, 4, 7, 11). They also strengthen the view

(4, 7, 11) that normal hardening is the result of incorporation of calcium into the outer pectic layer of the elongating cell walls, and is not due to changes in the cellulosic substances alone (9). With the gradual change to calcium pectate in the region of new growth, the short cells develop long hairs, while the long cells retain their normal shape and position in long rows.

Acknowledgment

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CORRECTION

Volume 34, page 459.

In line 21 of the Abstract, "Asparagine, for example, ——" should read "Arginine, for example, ——".

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Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

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An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

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